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Final Comprehensive Report

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PREPARATION OF A HOMOLOGOUS (HUMAN) INTRAVENOUS BOTULINAL IMMUNE GLOBULIN

FINAL COMPREHENSIVE REPORT

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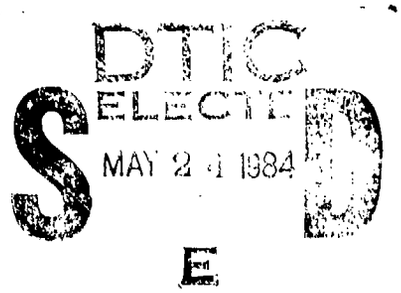

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An intravenous, botulinal, immune (human) globulin has been prepared from human immune plasma supplied by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick, Frederick, Maryland, and contains neutralizing activity to five botulinal neurotoxins: A, B, C, D and E. This final report contains details of all source materials, methods, procedures, testing and status of four lots of human, intravenous, hyperimmune, pentavalent, botulinal IgG that have been prepared and shipped to USAMRIID. These include lots IVBG-1A-553 grams, IVBG-1B--579 grams, IVBG-2A--563 grams, and IVBG-2B--401

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grams for a total of 2096 grams. The information included in this report contains all the documentation required of products prepared for Investigational New Drug (IND) status.

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II. Results

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Flow Sheets, Tables, Figures and Appendices (continued)

Summary

Preparation of a homologous (human), intravenous, botulinal immunoglobulin from human immune plasma supplied by United States Army Medical Research Institute of Infectious Diseases (USAMRIID) has been completed. A total of 2753 units, constituting a total of 937.7 liters of plasma, were processed and the IgG isolated was found to contain neutralizing activity (IU) to five botulinal neurotoxins: A, B, C, D and E. Four lots of botulinal immune globulin (human) pentavalent (A, B, C, D and E) were prepared; lots IVBG-1A--553 grams, IVBG-1B--578 grams, IVBG-2A--563 grams and IVBG-2B--401 grams for a total of 2095 grams. Included in this report are detailed specifications and identification of all source materials, presentation of all methods used in the preparation of the final product, and details and status of all testing including on-going shelf-life studies on the final product lots.

The material has been prepared by new patented methods involving SiO_2 stabilization of plasma followed by isolation and purification of IgG by QAE A-50 Sephadex ion exchange chromatography. The product is a monovalent, non-aggregated, non-fragmented, native, human IgG containing the IgG subclasses, IgG_1, IgG_2, and IgG_4. The human IgG contains neutralizing activity against botulinal neurotoxins A, B, C, D and E and the final product vials have neutralizing activity in IU/ml printed on the labels. This material has been prepared by methods used in this laboratory over the past 10 years for preparation of human, intravenous IgG that has been used clinically. Similar material has been administered intravenously in doses from 20 mg/kg to 400 mg/kg in over 400 patients for a total of over 1000 administrations with no evidence of severe reactions nor transmission of hepatitis. This material, on the basis of that experience and the extensive testing performed during its preparation, is considered safe for intravenous administration. Final

directions for dosage and administration have not yet been decided. It is recommended that it be stored at -20°C, and on the basis of previous work in our laboratory, should be stable for at least 10 years under the conditions of storage. The preparation is sterile, but contains no preservatives and therefore is supplied in single entry 10 ml vials. Testing including anti-complementary activity, half-life, and shelf-life studies are still in progress. IVBG-2A and IVBG-2B final products have been resubmitted for repeat subclass determination testing. Results of these tests will be communicated to USAMRIID when they become available.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Introduction

The purpose of this project was the preparation of an intravenous, pentavalent, botulinal, immune human IgG from human hyperimmune plasma. The plasma (937.7 liters) was obtained by USAMRIID by plasmapheresis of volunteers hyperimmunized to five specific botulinal neurotoxins: Types A, B, C, D and E. This material was shipped to the Minnesota ALG Program, Department of Surgery, University of Minnesota Medical School, Minneapolis, Minnesota by USAMRIID in July and August of 1981. Fractionation of plasma has been completed and the final product shipped to USAMRIID.

This comprehensive report details all procedures utilized, results and status of all testing specified in the contract proposal. The hyperimmune plasma was divided into four lots for fractionation. Each lot is identified by a specific lot number and individually tested for neutralizing activity, purity, sterility, pyrogenicity, safety, IgG subclass composition, temperature and storage stability, degree of fragmentation and aggregation, and proteolytic enzyme content. The four lots of sterile pyrogen-free intravenous, pentavalent botulinal immune globulin contain neutralizing activity against botulinal neurotoxins A, B, C, D and E and contain the major IgG subclasses IgG_1, IgG_2, and IgG_4. They all possess the structure and biological characteristics of native human IgG, i.e. M.W. 160,000, less than 1% aggregates, less than 1% fragments, are free of contaminating plasmin, stable on storage at -20°C or 4°C and considered safe for intravenous administration. Four lots of final product have been shipped to USAMRIID: Lot IVBG-1A, 553.11 grams; IVBG-1B, 577.8 grams; IVBG-2A, 562.8 grams; and IVBG-2B, 400.67 grams for a total of 2,094.38 grams of intravenous, pentavalent, botulinal, immune, human IgG.

The methods developed in our laboratory for the fractionation of this plasma are patented (Appendix A1, A2, A3) and have been used in the past to prepare intravenous normal human IgG (Appendix B). This material has been in clinical use since 1975. During this time period over 400 individuals have received IV doses of 20 mg/kg to 400 mg/kg. The material has been well tolerated and in over 1000 IV administrations, there have been three instances of elevated fever and no evidence of hepatitis transmission (1). In addition, we have prepared, by these methods, human intravenous hyperimmune cytomegalovirus (CMV) IgG for use in a randomized clinical trial to prevent CMV infection in high risk bone marrow transplant recipients. The results of this study show no evidence of adverse reactions, but more importantly, show a highly significant effect in prevention of CMV infection. There was no CMV infection, disease or mortality in the treated group (2,3,4,5).

I. Methods

A. Fractionation

The methods used to prepare a pentavalent, botulinal, immune globulin for safe intravenous use from human hyperimmune anti-botulinal plasma were adapted from procedures developed in this laboratory. These procedures are described in Appendices A1, A2, A3 and B. In order to maximize yields and purity, the procedures have been adapted to this specific application, i.e. changes in SiO_2 concentration, and use of an additional chromatography step. The material and methods used in the preparation of IVBG-1A, IVBG-1B, IVBG-2A and IVBG-2B are detailed in the following section and in the fractionation results section later.

1. Plasma Pool

Source - Two shipments of USAMRIID human botulinal immune plasma totalling 2753 units were received. This plasma had been collected, frozen, and shipped in 500 ml transfer packs. The first shipment of 1364 units was received on July 15, 1981, from the Michigan Department of Public Health. Approximately 50% of these units were damaged prior to or during shipping. The second shipment included 1389 units from USAMRIID, Fort Detrick, Frederick, Maryland, on August 12, 1981. This shipment was in very good condition with 10% of the units damaged.

Handling and Storage - The units were received in a frozen state. They were packed into new boxes, labelled, and moved to a -20°C storage facility before thawing occurred. As each lot was scheduled for production, one-half of a shipment (approximately one quarter of the total volume) was transferred from the cold storage facility to the fractionation facility.

Recording - The unit donor number, source, and date drawn were recorded at the time of thawing. This information is contained in Appendix C1, C2, C3 and C4 for lots IVBG-1A, IVBG-1B, IVBG-2A and IVBG-2B respectively.

Pooling - The transfer packs were rinsed in 70% ethanol, cut open, and the contents pooled into 50 gallon polyethylene tanks. All containers and instruments were either sterilized with ethanol, by

steam autoclaving, or sanitized with bleach and rinsed repeatedly with 80°C pyrogen-free water prior to use. Damaged transfer packs were identified, opened and the frozen material added directly to the tanks. The pooled plasma was thoroughly mixed with a motor-driven propeller. The volume was recorded and a plasma pool sample taken for testing. The plasma was maintained at 4°C throughout all of the following procedures except where noted.

2. Stabilization of Plasma

Addition of SiO_2 - Sterile, pyrogen-free, synthetic silicon dioxide (Aerosil 380, Degussa, Teterboro, NJ) is slowly added in a dry form to the plasma pool while mixing rapidly. Care is taken to avoid foaming. After the desired amount of silicon dioxide (SiO_2) has been added (20-30 grams of SiO_2 per liter of plasma) the mixture is stirred for one hour and then allowed to settle overnight at 4°C.

Removal of SiO_2 - SiO_2 is removed from the plasma by centrifugation. After settling in a tank overnight, the top layer of plasma (the supernate) contains very little SiO_2. This is pumped into a second tank for the final centrifugation step. The bottom layer (the sediment) in a settling tank contains most of the SiO_2 and some plasma. This sediment is dispensed into 1.0 liter sterile, polypropylene bottles for low speed centrifugation. This low-speed spin is done in Dupont-Sorvall RC3 refrigerated centrifuges with HG-4 swinging-bucket rotors (E.I. Dupont de Nemours and Co., Wilmington, DE) at 4°C, 5000 RPM, 6975 X g, for 45 minutes or in a Beckman J-6

centrifuge with a JS 4.2 swinging-bucket rotor (Beckman Instruments, Palo Alto, CA) at 4°C, 4200 RPM, 5010 X g, for 60 minutes. The supernate from this spin is added to the supernate drawn off earlier from the settling tank. The pellet from the low-speed spin consisting of packed SiO_2 and a small amount of occluded protein is either discarded or in some cases, resuspended with normal saline and spun again to recover some of the occluded protein. This additional step is referred to as an SiO_2 cake wash and provides some marginal increase in yield.

All supernates are combined and pumped into a Beckman J2-21C, J2-21 or a J2-21M refrigerated high speed centrifuge equipped with a JCF-Z continuous-flow rotor spinning at 18,000 RPM, 32,260 X g, temperature controlled at 4°C, with a feed rate of 250 ml/min/rotor. This final centrifugation step removes residual SiO_2 particles.

3. Filtration

The stabilized plasma is sterile filtered to remove any bacteria introduced during earlier processing. Filtration is accomplished by placing the stabilized plasma in stainless steel pressure vessels, pressurizing to 10-20 psig with medical grade nitrogen, (Nitrogen N.F., Ohio Medical Products, Madison, WI) and feeding the pressurized plasma to a double high stainless steel filter housing of sanitary design (Pall SANN2G723, Pall Trinity Micro Corp., Cortland, NY) containing a 20 inch prefilter. The filtrate from

this filter directly feeds a final filter housing of similar design (Pall SANN1G723) and an absolute C.2 micron pore size filter that has been autoclaved and aseptically attached to several closed, autoclaved polypropylene 15 gallon tanks. The entire plasma volume is passed through this system and is thus rendered sterile and held at 4°C until the next procedure.

The filter assemblies used in production are all flushed with 80°C pyrogen-free water prior to use or autoclaving. Table I lists the manufacturer's part number and the IVBG lot number in which each filter was used. This is a list of filters used at all steps in production including sterile filtration of plasma, final product, diafiltration buffers, and chromatography buffers. All filters used are pharmaceutical grade, non-fiber releasing and are manufactured in accordance with current good manufacturing guidelines.

4. Concentration and Buffer Exchange

Ultrafiltration System - The protein concentration and buffer composition of the protein solutions at various steps in fractionation are adjusted by concentration and diafiltration procedures using an ultrafiltration system. This system consists of a stainless steel, sanitary design, Millipore Pellicon high volume molecular filtration cell (Millipore Corp., Bedford, MA) equipped with 50 square feet of type PTGC (Millipore) 10,000 molecular weight cut-off filters. The system is fed by a stainless steel sanitary lobe pump (Waukesha Model, 10D0, Waukesha Div., Abex Corp., Waukesha, WI). The system

operates at 4°C, with a total flow rate of 4-8 liters per minute at 30-50 psig.

Concentration - Following sterile filtration and QAE procedures, the total protein concentration averages 33 mg/ml and 6.3 mg/ml respectively. The protein concentration must be increased after each of these procedures. This is accomplished by recirculating the protein solution through the ultrafiltration system under pressure and discarding filtrate until the desired volume or concentration of the protein solution is reached. This concentration ranges from 50 mg/ml for an IgG/QAE purification step, to 80 mg/ml for a plasma/QAE purification step, to 100 mg/ml for the final product. For a diafiltration procedure the protein is first concentrated to 50-60 mg/ml and at the end of diafiltration concentrated or diluted to one of the concentrations described above.

Buffer Preparation - Various buffers are used during fractionation primarily to adjust the pH, conductivity and ionic composition of the protein solution. All buffers are prepared in autoclaved polypropylene tanks or in polyethylene tanks sanitized with 1% bleach and rinsed six times with 80°C pyrogen-free water. Water for preparation of buffer is purified by deionization, followed by distillation in a Barnstead 30 gallon per hour Century Cyclone still (Barnstead Co., Boston, MA) with storage at 80°C, and is sterile and pyrogen-free. The chemicals used to make up all buffers used in fractionation are listed below along with the manufacturer from which they are obtained:

Sodium Chloride		Mallinckrodt Inc., Paris, KY
	or	Spectrum Chemical Mfg. Corp., Gardena, CA
Sodium Acetate		Mallinckrodt Inc.
Imiodazole		Sigma Chemical Co., St. Louis, MO
Glacial Acetic Acid		MCB Manufacturing Chemists, Inc., Cincinnati, OH
	or	Mallinckrodt, Inc.
	or	Spectrum Chemical Mfg. Corp.
190 Proof Ethanol		Worum Chemical Co., St. Paul, MN
Sodium Hydroxide		Fisher Scientific Co., Fairlawn, NJ
Sodium Carbonate		Sigma Chemical Co.
Sodium Bicarbonate		Sigma Chemical Co.

Buffers are sterile filtered as they are used.

Conductivity and pH Measurements - Each buffer is tested for pH and conductivity and must be within certain limits before use. Typically, the acceptable range for pH is $\pm$0.05 units and for conductivity, $\pm$0.1 milli Siemens (mS). Measurements are made on a Radiometer model CDM3 conductivity meter (Radiometer, Copenhagen, Denmark), Radiometer model pH23 pH meter or a Beckman model 3500 (Beckman Instruments, Irvine, CA) pH meter.

Diafiltration - Protein solutions are diafiltrated prior to anion exchange chromatography to adjust pH, conductivity and ionic composition. The product from the chromatography procedure is diafiltrated to replace the buffers used in chromatography with salts suitable for storage and administration of the final product. Diafiltration is done on ultrafiltration equipment (described earlier in the section) by adding the replacement buffer at the same rate as filtrate is produced by the system. This procedure is also called "constant volume washing". A volume of buffer equal to five times the volume of the protein solution is passed through the protein solution in this manner. The exchange of buffer after five volumes is 99% complete. Prior to anion exchange chromatography the buffer used is pH 6.6, .0194 M imidazole, 0.0888 M acetate, conductivity 5.85 mS at 21°C. This same buffer is used to equilibrate the anion exchange resin. The final product is diafiltrated with normal saline (0.154 M NaCl). Because saline has no buffering capacity the pH of the protein solution is raised during diafiltration by titration of the protein with small amounts of 1 N NaOH or Delory and Kings carbonate-bicarbonate buffer added to the saline.

5. Anion Exchange Liquid Column Chromatography

Anion exchange chromatography (QAE) is used to isolate a pure IgG fraction from the stabilized plasma or from a partially purified IgG fraction. A column chromatography system consisting of four 16 liter stacks (Series KS370/15, Pharmacia, Piscataway, NJ) con-

nected in parallel-flow configuration and packed with a total of 2.6 to 3.2 kg of QAE A-50 Sephadex (Pharmacia) is used.

The entire system is sterilized with a pH 6.6 buffer containing 0.0132 M imidazole, 0.0592 M acetate, 29.4% ethanol with a conductivity of 2.05±0.05 mS at 21°C. Just prior to use the QAE is equilibrated with a minimum of five volumes of a sterile pH 6.6, .0194 M imidazole, 0.0888 M acetate buffer with a conductivity of 5.85 mS at 21°C. All buffers applied to the stacks are filtered through sterile 0.2 micron filters. For a plasma run, (QAE 1) the plasma is spun in Beckman J21-C, J2-21 or J2-21M centrifuges equipped with JCF-Z continuous-flow rotors spinning at 18,000 RPM, 32,260 X g, with a throughput of 250 ml/min/rotor and a temperature setting of 21°C. This step removes particulates and warms the plasma for greater ion exchange efficiency.

For a QAE 2 run the product from a QAE 1 (plasma run) is concentrated to 50 mg/ml and then applied to one 16 liter stack.

Plasma or IgG is applied to the stacks in parallel by gravity feed. The flow rate through the stacks is controlled at 150 ml/min/stack with flow meters. Under these conditions only IgG passes through the anion exchange resin without binding. The effluent from the stacks is collected in sterile four liter cylinders (1.0 liter for an IgG run) with samples being taken from every cylinder for protein determination. After all the protein has been applied to the stacks, a sterile pH 6.6 (imidazole-acetate 5.85 mS at 21°C) buffer

is used to elute the remaining IgG from the QAE. Collection of the effluent is terminated at the point where proteins other than IgG begin to elute.

The collected protein from one or more plasma runs is pooled in sterile tanks. If the IgG contains measurable quantities of alpha-2-macroglobulin, transferrin or lipoproteins, it is concentrated and passed over an additional QAE stack. When the IgG is >99% pure, it is concentrated to the desired concentration and diafiltrated with normal saline using ultrafiltration procedures described previously. The final product (protein concentration of 103-108 mg/ml) is adjusted to pH 6.7-7.4. The product is spun in a Beckman J2-21C, J2-21 or J2-21M centrifuge with a JCF-Z continuous-flow rotor at 20,000 RPM, 39,260 X g, 200 ml/min/rotor, 21°C to remove particulates and aggregated protein and to warm the solution for proper functioning of the bottling equipment. Conductivity, pH, and protein concentration are checked and the solution is delivered to the testing group for filtration and bottling.

B. Bottling, Storage, Labels and Shipping

1. Bottling

Immediately after the high speed spin, the final products are prefiltered through a 0.2μ Pall Sealkleen cartridge. The material is forced through the filter by placing the product in a stainless steel pressure vessel and pressurizing with medical grade nitrogen

as described earlier. The IgG preparations are then sterile filtered by the same method through an autoclaved Pall DFA 0.2μ final filter system into a sterile 4 liter Erlenmeyer flask.

At approximately the same rate that the product is sterilely filtered into the final filter flask, it is drawn from the flask and aliquoted into sterile 10cc vials (Cat. #223739, Wheaton, Mellville, New Jersey). The material is dispensed, 10cc per vial, by a semiautomated bottling apparatus consisting of a National Instrument's Filamatic pump (#DAB-16, National Instrument, Baltimore, Maryland) attached to a calibrated sterile stainless steel syringe with a bottling bell attached.

Two technicians perform the bottling under a Baker Edge Gard laminar flow hood (Baker, Sanford, Mass.). They wear sterilized caps, gowns, masks and gloves, and observe sterile technique throughout the bottling procedure. Each vial is hand stoppered (Cat. #S-46, West Co., Phoenixville, PA or Cat. #13237, Pierce, Rockford, Illinois) immediately after filling. All vials are hand crimped (Cat. #20-10 West Co.) before being removed from the hood.

2. Storage

The bottled final product materials were stored at 4°C for 4 to 7 months until they were labelled and stored at -20°C. Stability studies conducted in our laboratory suggest that IgG purified by our method can be stored for several years at -20°C, 4°C or room temperature with no fragmentation and without loss of potency (6).

3. Labels (Appendix H)

In accordance with CFR 610.60, each vial has been labelled with a custom designed label describing the name of the product, name and address of the manufacturer, lot number, protein concentration and recommended individual dose. Antibody titers (IU/ml) to botulinal toxins A through E are printed on each label.

After IVBG-1A was shipped to Fort Detrick, the label for the next three lots was modified to include the following statement:

> Caution: New Drug - Limited by Federal Law to Investigational Use.

An actual label used for each lot is affixed to the page in Figure 1.

4. Shipping

When all testing was completed, each of the four lots of the antibotulinal toxin final products were shipped to Fort Detrick. Only those vials required for testing purposes have been retained at the University of Minnesota.

The final products were shipped as soon as possible after each respective lot was labelled. The vials were placed in custom made, printed, individual cardboard boxes. These boxes were then packed with dry ice in Styrofoam shipping containers and shipped on overnight services via either Federal Express or Emery Air Freight. A confirmation of delivery to Fort Detrick was received for each shipment.

C. Testing

1. Sterility, CFR 610.12 (Appendix H)

Twenty vials of each lot of the final products are selected at random to represent all stages of bottling. These are numbered 1-20 and submitted to Economics Laboratory, Inc. (Mendota Hts., Minn.) for sterility testing. In accordance with CFR 610.12, the contents of each vial are innoculated into thioglycollate medium and soybean casein digest medium and incubated for 14 days at 30-32°C and 20-25°C respectively. These test media are observed for growth on days three, four, seven, eight and fourteen. This regulation requires that no growth appear in any of the cultures for the material to pass the sterility testing. Economics Laboratory, Inc. submits written reports of each lot tested.

2. Pyrogen Test, CFR 610.13b (Appendix H)

Each lot is tested for the absence of pyrogenic substances in accordance with CFR 610.13b, as follows.

Three to four conditioned New Zealand White rabbits (Dutchland Laboratory, Denver, Penn., 3-5 lbs., females) are injected intravenously with final product material. Each rabbit receives a dose of 1 ml per kilogram via an ear vein and their temperatures are monitored hourly for three hours. Prior to injection, a normal baseline temperature is established and recorded. For the products to pass, no rabbit's post injection temperature can deviate 0.6°C or higher from its baseline temperature.

3. General Safety Test, CFR 610.11 (Appendix H)

To determine general toxicity the final products are tested as described in CFR 610.11. The final products are warmed to 37°C and injected intraperitoneally into two guinea pigs (Gopher State Caviary, White Bear Lake, Minn. <400 grams each) and two mice (Bio Labs, St. Paul, Minn., <22 grams each). Each guinea pig is injected with 5cc of product, each mouse with .5cc. The animals' weights are recorded at the beginning of each test. The animals are observed each working day for seven days and their weights recorded on day seven. To pass the requirements of the General Safety Test, all the animals must remain overtly healthy and lose no weight during the test interval.

4. Hepatitis, CFR 610.40 (Appendix H)

In accordance with CFR 610.40 each lot of final product is tested for the presence of hepatitis B surface antigen and for antibody activity against this antigen. Five milliliters of each lot is submitted to the University of Minnesota Blood Bank for testing by a radioimmuno assay (Ausie II and Ausab kits, Abbot Laboratories, Plymouth, Minn.). A written report is submitted to the laboratory for each lot.

5. Neutralizing Activity

The antibotulinal neutralizing activity of each lot is determined at USAMRIID by Lt. Colonel George Lewis and Major Martin Crumrine. Five to ten vials of each lot were shipped to Fort Detrick shortly after bottling for in vivo neutralizing activity titering against toxin types A, B, C, D, and E. A description of the procedures used for testing can be obtained from USAMRIID.

6. Immunoelectrophoresis (IEP)

The purity and electrophoretic mobility of the final products are analyzed by immunoelectrophoresis. In immunoelectrophoresis, two methods are combined--electrophoresis of the samples in an agarose matrix, followed by immunodiffusion and reaction with antiserum specific for human immunoglobulins or whole human serum.

Agarose gel (1%) IEP plates (IEP Kit #912, Kallestad Laboratories, Austin, Texas) are used for all sample analyses. These are pre-cut plates, each containing 7 sample wells divided by 6 antiserum holding troughs. Sodium barbitol IEP buffer is also supplied in the kit. This is dissolved in distilled water, pH 8.6.

- Undiluted samples of the final product materials are placed in the wells, 3 µl per well. The IEP plates are then placed in a Behring IEP Electrophoresis Chamber (Cat. #23-705-301, Behring, San Diego, CA), filled with IEP buffer. The samples are electrophoresed for 75-90 minutes at 80 volts using a D.C. Power Supply (Cat. #3371E, LKB, Gaithersburg, MD).

The plates are then removed from the chamber and the troughs are filled with one of the following antisera: rabbit anti-whole human serum, rabbit anti-human IgG, or goat anti-human total immunoglobulin.

The antiserum is allowed to diffuse into the agar to react with the separated specific proteins for 18 hours at room temperature. The plates are then blotted and pressed for two hours, followed by washing in two changes of NaCl to remove unreacted proteins. They are then washed 18 hours in distilled water to remove the salts, dried, and stained with .5% Coomassie Blue Stain.

7. Analytical and preparative Isoelectric Focusing (IEF)

Analytical Isoelectric Focusing - Samples are focused in a linear pH gradient from 3.5 to 9.5 on pre-cast gels (Cat. #1804-101, LKB). The gels contain 2.4% (w/v) ampholyte, 5% (w/v) polyacrylamide, are 3% cross-linked and are 235 X 90 X 1 mm in size. Equipment used includes a multiphor focusing unit (Model #2117, LKB), power supply, (Model #2103, LKB) and recirculating cold bath (Model #2209, LKB).

Samples (20 µl containing 0.2 to 2.0 mg protein) are applied onto filter paper tabs (5 X 10 mm, Whatman, Paratex) which are placed approximately 2 cm from the cathode. The cathode and anode electrode solutions are 1.0 M NaOH and 1.0 M H_3PO_4 respectively. The samples are focused for 90 minutes, 4°C, at 30 watts constant power. The application tabs are removed after 45 minutes.

At the end of the run, the pH gradient of the gel at 4°C is determined. A Beckman 3500 pH meter (Beckman Instruments, Irvine, CA) with a combination surface electrode (Cat. #2117-111, LKB) is used to determine the pH of the gel in 1 cm increments. The gel is focused for 10 additional minutes to increase the sharpness of the bands. Alternately, pH standards (pH Kit 3-10, Pharmacia, Piscataway, New Jersey) are included in the gel and the gel is allowed to reach 20°C during the last 20 minutes of the focusing run.

The focused gel is fixed for 45 minutes in a solution of 1% methanol (v/v) and 0.5% sulphosalicylic acid. Prior to staining, the gel is soaked for 5 minutes in destaining solution which contains 25% ethanol (v/v) and 8% acetic acid (v/v). This allows the gel to equilibrate to the staining conditions and also helps to remove ampholytes. The gel is stained for 10 minutes at 60°C in a filtered stain consisting of 0.1% Coomassie Blue R-250 (w/v) in destaining solution. The gel is destained for at least 48 hours with several changes of destain. To preserve the stained gels, they are soaked in destaining solution containing 10% glycerol (v/v) for 45 minutes, air dried overnight and covered with a thin plastic film.

Preparative Electrofocusing in a Granulated Gel (PEGG) - PEGG is performed in a glass tray (244 X 108 X 5 mm) containing a 100 ml slurry of protein (60-150 mg), highly washed G-75 Sephadex (4.0 gm), ampholytes (5% w/v) and distilled water. The protein is generally

dialyzed against 1% glycine prior to focusing to reduce the salt content. The ampholyte mixture is chosen to give a linear pH gradient throughout the pH range of interest.

The slurry is poured into the glass tray and air dried, with a 4" muffin fan, to an optimal water content. The cathode and anode wicks are soaked in 1.0 M NaOH and 1.0 M H_3PO_4 respectively and are placed along the short sides of the tray.

Samples are focused for 16-18 hours, 10°C, at a constant power of 8 watts. Voltage is set not to exceed 1,200 volts. Equipment used includes a multiphor focusing unit (Model #2117, LKB), power supply (Model #2103, LKB) and a recirculating cold bath (Model #2209, LKB). After the run is complete, a print is taken to record the pattern of the completed separation. A sheet of filter paper (Whatman #1, 224 X 103 mm) is applied directly to the gel surface for 30 seconds, removed and quickly dried with a hot air blower. The dry print is then washed three times in 10% trichloracetic acid for 15 minutes each time. The print is stained for 10 minutes, at room temperature, in a 0.2% Coomassie Blue R-250 solution (1:5:1 - methanol:water:acetic acid) and destained in the same solution without dye until the background color disappears. The print is then air dried.

The separated protein zones are collected by sectioning the gel bed into 30 equal parts using a fractionating grid (Cat. #90-00-0161, LKB). The pH gradient at 10°C is determined by placing a com-

bination surface electrode (Cat. #2117-111, LKB) directly into each section. Thirty small columns are placed over thirty polypropylene test tubes (Cat. #2006, 17 X 100 mm, Falcon, Oxnard, CA). Each gel section is transferred, using a spatula, to a small column and mixed with 1.5 ml 1% glycine. After the gel bed has settled, an additional 1.5 ml 1% glycine is added to the top of each column. The columns are centrifuged for 60 minutes, 280 X g, 4°C and the elutions collected. Total protein of each elution is determined by measuring the absorbance at 280 nm (Model 2600 spectrophotometer, Gilford, Oberlin, OH). Each elution may be tested further using radial immunodiffusion, enzyme assays, immunoelectrophoresis or analytical isoelectric focusing. Typically, 3 ml of eluant is recovered from each column and the total recovery of protein is 70-80%.

8. High Performance Liquid Exclusion Chromatography (HPLEC)

High performance liquid exclusion chromatography (HPLEC) is performed on a rigid, spherical, porous gel matrix. The matrix consists of silica particles of 10±2 micron size with a hydrophilic surface.

Ten microliters of sample (7-20 mg/ml) are applied to two 300 X 7.5 mm TSK-3000-SW columns (Beckman Instruments) connected in series. The columns are equilibrated in pH 6.3±0.1 PBS buffer (100 mM phosphate, 100 mM NaCl) at a flow rate of 0.5 ml/min. (Model 112 pump, Beckman Instruments). The column effluent is monitored for one hour at 280 nm (Model 2238 U.V. monitor, 2.5 mm flow cell,

0.05 AUFS, LKB) and a tracing is made of the separation (Model 2210 recorder, 0.5 cm/min., LKB).

The molecular weight of each peak is estimated using a linear regression formula based on the retention times of globular proteins of known molecular weight (Cat. #151-1901, Bio Rad Laboratories, Richmond, CA) ranging from 10,000 to 300,000 daltons. Proteins elute in order of decreasing size. The molecular weight of a sample is reproducible $\pm$5% over a six month period.

9. Protein Determination (Biuret, RID and Lipid)

Biuret - Total protein is determined by the Biuret method. Sample or standard, 50 ul, is added to 5.0 ml of normal saline (1:101 dilution). Biuret reagent (13.5 mM $CuSO_4$, 7.84 N NaOH), 2.5 ml, is added to the diluted protein, mixed and allowed to stand for 20-60 minutes before the absorbance at 310 nm (model 2600 spectrophotometer, Gilford, or Model 25 spectrophotometer, Beckman Instruments) is determined. A four point standard curve is constructed using bovine serum albumin (Cat. #81-016, Miles Laboratories, Inc., Elkhart, IN) standards at 20, 40 and 60 mg protein/ml and a blank consisting of 5.0 ml saline and 2.5 ml of biuret solution. All samples and standards are tested in triplicate and the average absorbance is used. The protein concentration of each sample is determined by using a linear regression formula based on the absorbance of the four standards.

Radial Immunodiffusion (RID) - Individual plasma proteins are quantitated by the Mancini method (6). Samples are placed in wells cut in agar containing specific antibody. When the specific antibody in the agar reaches equilibrium with its antigen, a ring of precipitate is established. At end point, the diameter of the ring is directly proportional to the protein concentration of the unknown which is determined by comparison with known standards. Ring diameters are measured at endpoint, to the nearest 0.1 mm using a calibrating RID viewer (Cat. #928, Kallestad Laboratories). High level albumin measurements, however, are timed (5 hours $\pm$ 15 minutes at room temperature). Standards are supplied by the plate manufacturers and immunoglobulin research preparations. A table of the RID plates used for this report is shown below:

Protein	Standard Range (mg/ml)	Lowest Detectable Level (mg/ml)	Manufacturer	Catalog Number
IgG	3.00-23.50	0.0460	KAL	519
IgG	0.15-3.50	0.0188	KAL	523
IgG	0.01-0.20	0.0060	KAL	525
IgA	0.45-4.25	0.0390	KAL	520
IgA	0.04-0.60	0.0076	KAL	527
IgM	0.45-4.00	0.0450	KAL	521
IgM	0.04-0.50	0.0252	KAL	530
Albumin	10.75-68.00	0.1000	KAL	640
Albumin	0.04-1.00	0.0160	KAL	567
Transferrin	0.60-4.50	0.0153	KAL	533
α_2-Macroglobulin	0.50-4.00	0.1000	KAL	Special Order
Hemopexin	0.30-1.46	0.1000	CALBIO	12-751-001
α-Lipoprotein (HDL)	0.10-0.40	0.0200	MD Lab	Special Order
β-Lipoprotein (LDL)	0.75-7.60	0.1000	MD Lab	Special Order

KAL - Kallestad Laboratories

CALBIO - Calbiochem-Behring, La Jolla, California

MD Lab - Mike Dolman Lab, Richfield, Minnesota

Lipid Analysis - Cholesterol and triglyceride levels are determined on a Technicon Autoanalyzer II. Assays are performed by the Central Lipid Laboratory, Department of Surgery, University of Minnesota in Minneapolis.

Cholesterol is determined by Technicon method SE4-0016FH4. Cholesterol is extracted in isopropanol, added to a modified Lieberman-Buechard reagent, and the absorbance measured at 630 nm. Triglycerides are determined by Technicon method SE4-0023FE5. Triglycerides are extracted in isopropanol, saponified to glycerol, and oxidized to formaldehyde. Triglycerides are then measured as formaldehyde by the formation of a flourescent condensation product (Hantzsch reaction). The lowest detection levels are 0.05 mg/ml and 0.10 mg/ml for cholesterol and triglycerides, respectively.

10. Assay Method for IgG Subclasses

Quantitation of IgG subclasses is accomplished by using a double antibody, radio labelled I^{125} assay. Assays are performed by Dr. William Yount, Dept. of Medicine, University of North Carolina at Chapel Hill. Samples are diluted from 1/1000 to 1/100,000. The specific IgG subclass antibody is added and incubated overnight. The I^{131} labelled subclass IgG (purified from human IgG subclass monoclonal myeloma sera) is added and incubated for two hours. Following this incubation, the second antibody is added and incubated overnight. The precipitate is washed three times and radioactivity measured and compared with a standard. For human

subclasses IgG_1 and IgG_3, the first antibody is a rabbit anti-human IgG_1 and IgG_3, and the second antibody is a goat anti-rabbit IgG. For human subclasses IgG_2 and IgG_4 and the second antibody is a goat anti-monkey IgG. The sensitivity and reproducibility of the method permits accurate measurement of as little as 0.0002 mg/ml of an IgG subclass.

11. Plasminogen Assay

Chromogenic substrate S-2251 (Kabi Diagnostica, Stockholm, Sweden) is a synthetic peptide which is used for the detection of the enzyme plasmin. When S-2251 is reacted with plasmin, a chromophore is released causing a spectrophotometric increase at 405 nm. The substrate is sufficiently specific and sensitive to measure 0.01 CTA[1] units/ml of plasmin. Trypsin and glandular kallikrein will react with S-2251 but to a lesser extent than plasmin. Most enzymes (including thrombin, factor Xa, plasma kallikrein and urokinase) have little or no effect on this substrate. The assay involves the addition of streptokinase (Cat. #S-3134, Sigma, St. Louis, MO) to diluted plasma samples which results in a plasminogen-streptokinase complex which has enzymatic activity. Since streptokinase is added in excess, no free plasminogen is left to be activated to plasmin and the complex is not inhibited by plasma inhibitors. Thus, we are

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[1] Committee on Thrombolytic Activity

measuring total plasminogen (potential plasmin) plus plasmin which is not bound to any inhibitor.

Our plasminogen standard is human plasma from eight donors. Equal volumes of plasma from the donors are mixed and dispensed into 2 ml aliquots. The plasma is stored at -57°C. The plasminogen content of the standard plasma (2.69 CTA units/ml) is determined from a standard curve prepared from purified plasmin (10.2 CTA units/ml, supplied by Dr. B. Alving, Bureau of Biologics, Bethesda, Maryland). On the day of each assay, 2 ml of standard plasma is thawed at 37°C and then kept on ice.

Samples and standards are diluted 1:41 in a pH 7.4 Tris buffer (50 mM Tris, 12 mM NaCl). Assays can be performed between pH 6.5 and pH 9.0. Each sample, 200 ul , is dispensed into a 12 X 75 mm borosilicate glass test tube and incubated for 5 minutes at 37°C. Streptokinase (10,000 IU/ml in H_2O, 37°C), 1000 ul, is added to each sample. Incubation continues for exactly 10 minutes. Substrate solution (0.86 mM S-2251 in pH 7.4 Tris), 700 µl, is added to each sample, mixed and monitored at 37°C, 405 nm (Model 2600 Spectrophotometer, Gilford) in pre-heated, 1 cm semi-microcuvettes.

A nine point standard curve is constructed ranging from 0.00 CTA units/ml to 4.04 CTA units/ml. The plasminogen concentration is plotted against the ΔA405/min. and a linear regression analysis is performed.

The reaction rate increases linearly with increasing concentration of plasminogen. The plasminogen concentration of each unknown is calculated by entering the ΔA405/min. of the unknown into the linear regression formula. The ΔA405/min. is the average of a five minute reading. For each sample, the absorbance at 405 nm is plotted to verify a linear rate increase throughout the entire 5 minute period.

When our standard plasma (2.69 CTA units/ml) is diluted 1:250 (0.01 CTA units/ml) followed by the 1:41 dilution in pH 7.4 Tris buffer, a ΔA405/min. of 0.001 is observed (0.005 over a five minute period) which is easily measurable on our instrument. Thus, the lowest detection level possible is 0.01 CTA units/ml. Repeated testing of the same sample yields results which are reproducible within $\pm$0.10 CTA units/ml.

12. Prekallikrein Assay

Substrate S-2302 (Kabi Diagnostica, Stockholm, Sweden) is used for the detection of plasma kallikrein. The plasma kallikrein formed catalyzes the splitting of the chromophore p-nitroaniline (pNA) from the substrate H-D-Pro-Phe-Arg-pNA. The rate at which pNA is released is measured photometrically at 405 nm. The substrate is sufficiently specific and sensitive to measure 0.1 μg/ml of plasma kallikrein. Other enzymes, including plasmin, will react with S-2302 but to a lesser extent than plasma kallikrein. The assay involves the activation of prekallikrein to kallikrein by an activator (Plasma prekallikrein activator, Kabi Diagnostica) consisting of

a mixture of ellagic acid, cephalin, Hageman factor and high molecular weight kininogen in Tris-HCl buffer (50 mM, pH 7.8). By using citrated plasma in a diluted system and a short activation time, the method is only slightly influenced by inhibitors of kallikrein. Thus, we are measuring the total prekallikrein (potential kallikrein) plus free kallikrein which is not bound to any inhibitor.

Our prekallikrein standard is human plasma from eight donors. Equal volumes of plasma from the donors are mixed and dispensed into 2 ml aliquots and stored at -70°C. The prekallikrein content of the standard plasma is 30.0 ug/ml. On the day of each assay, 2 ml of standard plasma is thawed at 37°C and then kept at room temperature.

Samples and standards are diluted 1:61 in a pH 7.8 Tris buffer (50 mM Tris-HCl). Assays are performed at pH 7.8±0.2. Activator, 200 µl, is incubated at 37°C in a 5 ml plastic test tube for 3 to 4 minutes. Standards or samples, 200 µl, are added, mixed and incubated at 37°C for 2 minutes. Substrate solution (2 mM S-2302 in pH 7.8 Tris), 200 µl is added to each sample, mixed and monitored at 405 nm (Model 2600 Spectrophotometer, Gilford), 37°C, in pre-heated, 1 cm semi-microcuvettes.

An eight point standard curve is constructed ranging from 0 µg/ml to 37.5 µg/ml. The prekallikrein concentration is plotted against the ΔA405/min. and a linear regression analysis is performed. The reaction rate increases linearly with increasing concentration of pre-

kallikrein. The prekallikrein concentration of each unknown is calculated by entering the ΔA405/min. of the unknown into the linear regression formula. The ΔA405/min. is the average of a two minute reading. For each sample, the absorbance at 405 nm is plotted to verify a linear rate increase throughout the entire 2 minute period.

When our standard plasma (30.0 μg/ml) is diluted 1:250 (0.12 μg/ml) followed by the 1:61 dilution in pH 7.8 Tris buffer, the ΔA405/min. is easily measurable on our instrument. Repeated testing of the same sample yields results which are reproducible within ±.20 μg/ml.

13. Circulatory Half-Life

It was initially proposed that the circulatory half-life of the IVBG final products be determined by our laboratory. Testing was done by intravenously injecting normal rabbits with final product and then at given intervals, serum samples were drawn from each animal. Anti-human gamma globulin radial immunodiffusion plates (Cat. #525, Kallestad Labs.) were used to quantitate the level and reduction of circulating human IgG in the rabbits' sera over time.

It was later decided that the half-life studies be conducted by USAMRIID in monkeys and human volunteers. The half-life of the neutralizing activity, not the circulating IgG will be measured by their method.

14. Anti-Complementary Activity

Samples of each final product are being tested for the presence of anti-complementary activity by Dr. Henry Gewurz, department of Immunology, Rush-Presbyterian, St. Luke's Medical Center, Chicago, Illinois. An enzyme assay will be used to determine the amount of final product protein required to bind 50% of the Clq in a standard solution.

15. Limited Proteolysis of IgG by Plasmin

Plasmin in an endopeptidase which cleaves arginine-lysine or valine-isoleucine peptide bonds. Human plasminogen (#51H304, 120 units/mg, Worthington Biochemicals, Freehold, NJ) is converted to plasmin by the addition of urokinase (#A18A, 1700 Plough units per vial, Leo Pharmaceuticals, Ballerup, Denmark). The ratio of Plough units to plasmin units is 2:1 at 9 mg/ml plasmin in a pH 8.0 Tris buffer (50 mM Tris, 100 mM NaCl, 20 mM glycine, 25% glycerol). IgG, plasmin, (38 mg:1 mg ratio) buffer and normal saline are mixed and incubated sterilely for 8 hours at 37°C. The final concentration of ingredients is 30 mg/ml IgG, 0.8 mg/ml (89 units/ml) plasmin, and 7 mM Tris-HCl (pH 8.0, 114 mM NaCl, 3 mM glycine, 4% glycerol). As a control, IgG samples are incubated at 37°C as above except that plasmin is omitted. Degradation is stopped by freezing the samples at -57°C. Anaylsis of the IgG degradation is done by HPLEC.

D. Shelf Life Study

For the purpose of studying the physical and biological stability of the final product preparations, shelf-life studies were initiated after each bottling.

Thirteen to twenty vials of final product were placed at various temperatures and will be assayed at the time intervals listed below:

Temperature	Storage Time
37°C	6 months, 18 months, 36 months
room temperature	6 months, 18 months, 36 months
4°C	6 months, 18 months, 36 months
-20°C	6 months, 18 months, 36 months

These will be assayed for the presence of split products by HPLEC, immunoelectrophoresis and isoelectric focusing. Levels of prekallikrein and plasminogen will be determined.

As close as possible to the dates listed below, an aliquot of each vial will be sent to Fort Detrick for determining the neutralizing antibody activity to botulinal toxins, A, B, C, D and E.

IVBG-1A	IVBG-1B	IVBG-2A	IVBG-2B	
4/28/82	11/10/82	2/04/83	3/03/83	6 month samples
4/28/83	11/10/83	2/04/84	3/03/84	18 month samples
10/10/84	5/10/85	8/04/85	9/03/85	36 month samples

All data obtained will be collated and sent to Fort Detrick. A copy will be filed in the ALG Laboratory IVBG log book.

II. Results

A. Fractionation

The two major steps in plasma fractionation are silicon dioxide treatment (SiO_2) and QAE A-50 sephadex anion exchange chromatography. SiO_2 removes or substantially reduces concentrations of plasminogen, prekallikrein, triglycerides, cholesterol, alpha-lipoprotein, beta-lipoprotein, clotting factors, fibrinogen and complement components in the plasma pool. The effect of SiO_2 is demonstrated by comparing the concentration of these plasma components in the plasma pool with the concentration at the pre-QAE stage. The QAE procedure isolates IgG from the remaining protein. The effect of QAE is demonstrated by comparing the concentration of various proteins in the pre-QAE stage with the final product. The SiO_2 and QAE procedures are the only procedures that substantially change the relative concentration of various plasma components. Other procedures remove particulates, exchange buffers, or change the concentration of all macromolecular components equally. For this reason the data presented in this section refers to plasma pool, pre-QAE (post SiO_2) and final product (post QAE) stages of fractionation.

Variables that affect the efficiency of these procedures are carefully controlled and monitored. When necessary, adjustments in these variables are made. The variables that affect the SiO_2 and QAE procedures, the results of these procedures and sequence of fractionation steps for each lot are presented in this section.

1. Flow Sheets

The general procedures used in the preparation of the four lots IVBG-1A, IVBG-1B, IVBG-2A and IVBG-2B are described in the Fractionation Methods section and are shown in Flow Sheet 1. Flow Sheets 2, 3, 4 and 5 for IVBG-1A, IVBG-1B, IVBG-2A and IVBG-2B respectively, show in greater detail the sequence and condition of the fractionation procedure, as well as the volumes, grams total protein and grams of IgG. Differences in the procedures from lot to lot apparent in the flow sheets result from changes made to maximize yields or ensure purity of the final product. For example, the SiO_2 concentration of 28 gm/l in IVBG-1A was increased to 30 gm/l to obtain complete removal of triglycerides before the QAE step. On IVBG-1A a second QAE procedure was done (QAE 2) to ensure complete removal of alpha-2 macroglobulin; on the following lots this QAE 2 step was not necessary. The difference in sterile filtration volmes of 302 liters for IVBG-1A and 209 liters for IVBG-1B was the result of adjustments made in the SiO_2 cake wash procedures. In Flow Sheet 3 for IVBG-1B two extra steps are shown. First, the addition of IVBG-1A remnants represents IgG produced in the fractionation of IVBG-1A by not bottled with that lot. Second, the "second bottling" of IVBG-1B was done because a white ring of protein had formed around the vial stoppers during storage at 4°C. The reason for these adjustment or modifications of procedures and the results of the major fractionation procedures are presented in this section.

2. Plasma Pool (Table II)

At the time of processsing, our facility was best suited for handling lot volumes of 200-300 liters. For this reason the plasma was processed in four lots of nearly equal volumes. Plasma pool volumes ranged from 230 liters to 240 liters with from 682 to 699 units being used in each lot. The plasma pool volume, the number of units, the source of the plasma and the date of plasma receipt of each lot is shown in Table II.

As the plasma units were thawed two things were noted. First, many of the transfer packs were damaged and plasma had started to leak from these bags. The incidence of broken bags was much higher in the shipment from Michigan (approximately 50%) than in the USAMRIID shipment (approximately 10%). Second, all of the plasma was highly lipemic, more so than the cryopoor plasma that we usually process.

3. SiO_2 Treatment

When a fumed silica powder such as Aerosil 380 is added under controlled conditions to plasma, certain plasma components are preferentially adsorbed onto the surface of the silica particles. The particle and adsorbed protein complex may then be removed by sedimentation. In general, all proteins are adsorbed to some extent but some are so efficiently bound as to be quantitatively removed by this procedure.

A discussion of some of the effects of silica on the treatment of plasma in the preparation of organ perfusion solutions may be found in Appendex A1.

Unwanted plasma components that are removed or substantially reduced by SiO_2 treatment are plasminogen, prekallikrein, triglycerides, cholesterol, alpha-lipoprotein, beta-lipoprotein, clotting factors, fibrinogen and complement components. Removal of these proteins results in a highly stable, clarified protein solution.

In the application of this procedure to the treatment of the IVBG plasma pools, the high level of lipids had to be considered in order to determine the amount and the conditions of the addition and processing of the SiO_2. The addition of two or three small amounts of SiO_2 with the removal of SiO_2 and adsorbed proteins after each addition is known to give better yields than the addition of the same total amount of SiO_2 at one time. Also, it has been shown recently in our lab that lowering the pH down to 5.5 requires less SiO_2 to achieve the desired removal of unwanted components and thus gives higher IgG yields.

F. IVB. the plasma was adjusted to pH 5.5 with 6 molar acetic acid and three SiO_2 additions were done. After the first addition of 18 grams of SiO_2 per liter of plasma pool volume and the second addition . gm/l, the cakes were washed twice and the recovered protein set aside. A final 5 gm/l addition was done and all the protein was combined and centrifuged. Because this combination of con-

ditions did not completely remove triglycerides from the plasma pool (0.13 mg/ml remaining in the pre-QAE sample, see Table III), the IVBG-1B pool was not acidified and was treated with two SiO_2 additions of 20 gm/l and 10 gm/l to ensure the complete removal of unwanted components. The SiO_2 cakes were not washed on this lot. For lots IVBG-2A and 2B two SiO_2 additions of 20 gm/l and 10 gm/l were done on each with the SiO_2 cakes from the 10 gm/l addition being washed twice. Protein recovered from the washes was added to the final post SiO_2 centrifugation as in IVBG-1A.

Removal of Lipoprotein (Table III) - Removal of the lipid components in the plasma pool is important in several respects. The presence of high concentrations of lipids can make sterile filtration and ultrafiltration impossible. In addition, the QAE resin is not able to remove large quantities of lipids. If the correct amount of SiO_2 is added to the plasma, the lipids will be removed. For ordinary plasma, 20 gm/l is sufficient. For IVBG plasma pools, 30 gm/l was necessary. The lipid concentrations in the four IVBG plasma pools ranged from 0.55 mg of alpha-lipoprotein (HDL) per ml of plasma to 3.06 mg/ml for beta-lipoprotein (LDL). Triglyceride and cholesterol levels were intermediate within this range. Table III shows the levels of triglycerides, cholesterol, alpha-lipoprotein, and beta-lipoprotein in the plasma pool, pre-QAE and final product samples. The SiO_2 treatment reduced the levels of all four components to undetectable levels in every lot with the exception of IVBG-1A in which 0.13 mg/ml of triglyceride was still present in the pre-QAE sample. Anion exchange chromatography removed this residual amount.

Removal of Plasminogen and Prekallikrein (Table IV) - Plasminogen and prekallikrein are proenzymes which, in the presence of activators, can degrade other proteins. For this reason they must be removed. When plasminogen was measured in IVBG plasma pools, concentrations of 3.06 to 3.52 CTA U/ml were found. This represents as much as 841,000 total units of activity (IVBG-2B). Following SiO_2 treatment, plasminogen was not detected in any samples.

Prekallikrein concentration in the plasma pool ranged from 4.1 to 11.2 micrograms/ml. SiO_2 treatment reduces the total amount of prekallikrein by an average of 88%. QAE reduces the remaining prekallikrein for a total average removal of 99%. The prekallikrein concentration in the final product ranged from 0.5 to 3.8 micrograms per ml. Table IV contains the complete set of values for both plasminogen and prekallikrein in the fractionation samples.

Effect on IgG and IgG Subclasses (Table V) - The yield of total IgG through the SiO_2 step averaged 67.3% with a maximum of 75% for IVBG-1B and a minimum of 53% for IVBG-2B. Treatment with SiO_2 consistently results in the nearly complete removal of the IgG_3 subclass. The IgG_3 values shown in Table V for IVBG-2A and IVBG-2B are far above the expected normal range and suggest an error in the determination. For this reason samples of IVBG-2A and IVBG-2B have been resubmitted for subclass determinations. For IVBG-1A and IVBG-1B the indicated subclass yields were normal. The average yield following SiO_2 treatment was 88.5% for IgG_1, 57.5% for IgG_2,

0.45% for IgG_3, and 91.0% for IgG_4. The total IgG grams and percent yields discussed here and shown in Table V are based on the amount of IgG produced in each lot without adjustment for the 73 gram carryover from IVBG-1A to the IVBG-1B bottling.

4. QAE A-50 Anion Exchange Chromatography (Table VI)

Anion exchange liquid chromatography on QAE A-50 Sephadex is used to isolate native IgG from the stabilized pre-QAE protein solution. A number of critical variables are carefully controlled to obtain the maximum efficiency, yield and above all, purity from this procedure.

pH and Conductivity - The most critical variables are the pH and conductivity of the plasma, QAE and buffers. At a pH of 6.6 and a conductivity of 5.85 mS at 21°C, all of the pre-QAE plasma components bind to the QAE except IgG. IgG molecules which have isoelectric points near or above neutrality pass through the column without interacting with the QAE. The pH and conductivity of the process is determined by equilibrating the QAE, diafiltering the plasma, and eluting the IgG off the QAE with a pH 6.6, 0.0194 M imidazole, 0.0888 M acetate buffer which has a conductivity of 5.85 mS at 21°C. A higher pH or lower conductivity results in loss of yield (increased binding of IgG). A lower pH or higher conductivity results in the elution of non-IgG proteins such as transferrin.

Load, gm protein/gm Sephadex - The next most critical factor is the ratio of total protein applied to the given grams of QAE in the column. If the quantity of protein that must be retained by the column exceeds the number of available binding sites, the column becomes overloaded and contamination of the IgG results. The first protein to break through under these conditions is alpha-2-macroglobulin. To avoid this, a maximum safe load is determined by experimentation and a load as close to this as possible is applied. Lower loads represent an inefficient use of materials. Loads used for the IVBG lots were restricted to a narrow range with maximum of 1.50 of IVBG-2A and a minimum of 1.37 on IVBG-2B.

Protein Concentration - The concentration of the protein applied to the column is also important. For a given load, a low concentration will require much larger volumes of protein to be applied. This increased volume may result in the elution of weakly bound contaminants from the resin. Also, higher applied concentration yields high IgG concentrations in the effluent resulting in less potential for low concentration induced denaturation. Protein concentrations above 80 mg/ml are too viscous for proper flow through the column. The concentration of protein applied to the column was carefully controlled to 80 mg/ml $\pm$ 0.7 mg/ml.

Table VI shows the actual protein concentration, total volume and ratio of protein to QAE or load applied to the column.

IgG and IgG Subclass Yields - Table VI shows the yield of total IgG through the QAE procedure in grams, as percent of the IgG applied, and as a percent of total protein applied. An average of 74.4% of the IgG applied to the QAE was recovered. The total IgG and IgG subclass yields for the main fractionation steps are shown in Table V. The final product yields of total IgG were 51%, 54%, 48%, and 34% for lots IVBG-1A, IVBG-1B, IVBG-2A, and IVBG-2B respectively. Final product samples for IVBG-2A and IVBG-2B have been resubmitted for subclass determinations. The average subclass yields for IVBG-1A and IVBG-1B were 67.5% for IgG_1, 42.0% for IgG_2, 1.2% for IgG_3, and 5.0% for IgG_4. As previously discussed the low yield of IgG_3 is due to SiO_2 treatment. The low yield of IgG_4 is a result of the QAE procedure which reduced IgG_4 concentrations an average of 94.3%.

Removal of Some Plasma Proteins - It should be noted that the pre-QAE plasma is always concentrated to 80 mg protein/ml as compared to 50 mg protein/ml of the starting plasma pool. The concentration of transferrin, alpha-2-macroglobulin, hemopexin and albumin were measured by RID in the three major fractionation samples from each lot (Table VII). It can be seen that hemopexin is reduced by SiO_2 treatment of the plasma pool. The other three proteins albumin, alpha-2-macroglobulin and transferrin are not reduced significantly by SiO_2. However, following column chromatography, hemopexin, alpha-2-macroglobulin, and albumin were not detectable.

Removal of IgA and IgM (Table VII) - The concentration of IgA, IgM, and IgG were measured in the fractionation samples from all four lots by radial immunodiffusion. This data is shown in Table VII. By comparison to IgG and IgA, IgM is selectively removed in part by SiO_2. This is demonstrated by the fact that the IgM concentration does not increase from the plasma pool sample to the pre-QAE sample as does the total protein and the IgG and IgA. Pre-QAE IgM concentrations average 0.81 mg/ml. After the QAE procedure, IgM was not detectable by RID. IgA, with an average pre-QAE concentration of 2.5 mg/ml, appeared to be removed only by QAE. However, the IgA radial immunodiffusion plates showed an unquantifiable precipitin ring for the final product samples of all four lots. This may represent the presence of IgA. Caution should be taken when IVBG is administered to individuals known to be deficient in IgA.

5. Total Fractionation Yields (Table IX)

Historically, fractionation of human plasma by the Cohn Method has yielded less than 40% of the IgG in the starting plasma. The primary purpose in using an ion exchange chromatography separation in this project was to produce an undenatured, native, intact IgG with high purity, good yields and which is suitable and safe for intravenous administration. Our method of fractionation has yielded at least 50% recovery of total IgG. The volumes, grams of total protein, percent yield, IgG grams, and percent yields from all four lots are summarized in Table IV and Figure 2.

The actual bottled yields of final product were 609.8 grams for IVBG-1A, 614.5 grams for IVBG-1B (rebottled), 622.7 grams for IVBG-2A, and 454.2 grams for IVBG-2B for a total of 2,301.2 grams.

These values are based on biuret determination. The total percent yields range from 5.46% (grams of final product bottled per 100 grams of plasma pool) protein for IVBG-1A to 3.83% for IVBG-2B.

The volumes, grams of total protein, total protein percent yield, IgG grams and percent yields for fractionation samples from all four lots are shown in Table IX. IgG determinations were done by RID and are less accurate than the biuret determinations.

Figure 2 presents the percent yield for total protein and IgG in ionic graph form. The final product values are not adjusted for the IVBG-1A and IVBG-1B bottling changes.

II. Results

B. Bottling, Storage, Labels and Shipping

1. Bottling Yields

The bottling dates and yields for each lot are indicated on Table X, Final Product Summary I. All filter types and lot numbers are also shown. These lots were bottled between October 1981 and August 1982. The protein concentration of the final products was 103-108 mg/ml. The total number of grams bottled was: IVBG-1A,

609.76, IVBG-1B, 614.5 (rebottled), IVBG-2A, 622.65, and IVBG-2B, 454.23. The total number of vials and grams that were available for shipment to USAMRIID equals the total number of vials and grams bottled less the amount of product required for testing purposes.

Lot IVBG-1B was rebottled seven months after the initial bottling. This was done because protein had dried during 4°C storage along the inside flange of the red rubber stopper. The vials were opened, the contents pooled, high speed spun and rebottled. This resulted in a 90 gram loss of product available for shipment due to handling and testing.

2. Storage, Labelling and Shipping

As soon as the antitoxin neutralizing activity had been determined and reported, the custom labels were printed with the appropriately reduced titers. The vials were then labelled and transferred from 4°C to -20°C storage.

IVBG-1A was stored at 4°C for 6 months before being labelled and frozen.

IVBG-1B was stored at 4°C for 7 months. It was then rebottled, labelled and frozen immediately.

IVBG-2A and 2B were stored at 4°C for 4 and 5 months respectively before being labelled and frozen.

Within two weeks of labelling and freezing, the final products were shipped to Fort Detrick. The shipment dates and number of vials and grams shipped per lot are listed below:

IVBG-1A	April 5, 1982	537 vials	553.11 grams
IVBG-1B	Jan. 4, 1983	535 vials	577.8 grams
IVBG-2A	Nov. 23, 1982	536 vials	562.8 grams
IVBG-2B	Jan. 11, 1983	389 vials	400.67 grams

These materials were packed on dry ice in Styrofoam boxes and shipped air, overnight service. As soon as they were received at Fort Detrick the materials were placed in the USAMRIID freezer facility.

II. Results

C. Testing

Several vials of each final product were used for testing purposes (34-56 vials, Table X).

As required by the FDA-CFR Part 610, Subpart B, General Provisions, the following various tests were performed using these vials to determine the sterility, safety, purity and potency of the final products. All the quality assurance and potency testing was completed before the products were shipped to USAMRIID. The results of these tests are shown on table XI, Final Product Summary II, Testing.

1. Sterility (Appendix D 1-4)

No growth appeared in any of the thioglycollate or soybean casein digest medium cultures. Therefore, all four lots were determined to be sterile. Reports submitted by Economics Laboratories, Inc. are found in Appendix D-1(IVBG-1A), D-2(IVBG-1B), D-3(IVBG-2A), and D-4(IVBG-2B).

2. Pyrogen Test (Appendix E 1-4)

As shown in Figures 3, 4, 5 and 6, no rabbit's temperature deviated 0.6°C or higher above its respective baseline temperature (as indicated by the dotted line of the bar graph). Therefore, these lots passed the test for the absence of pyrogenic substances. A copy of the pyrogen testing data sheets for each lot is found in Appendix E-1(1-4), E-2(IVBG-1B), E-3(IVBG-2A), and E-4(IVBG-2B).

3. General Safety Test (Appendix F 1-4)

All animals injected with these final products remained overtly healthy and gained weight during the test period. Each lot passed the requirements of the General Safety Test. Pre-test and post-test injection weights of the animals used for these tests are shown in Appendix F-1(IVBG-1A), F-2(IVBG-1B), F-3(IVBG-2A), and F-4(IVBG-2B).

4. Hepatitis (Appendix G 1-4)

Results of the hepatitis B surface antigen/antibody testing are shown in Appendices G-1(IVBG-1A), G-2(IVBG-1B), G-3(IVBG-2A), and G-4(IVBG-2B). All four lots were negative for hepatitis antigen and positive for anti-hepatitis antibody.

5. Neutralizing activity (Table XII; Figure 2)

The neutralizing activity (IU/ml) and specific activity (IU/mg) for each lot are seen on Table XII. All four lots were tested for activity against toxin types A, B, C, D and E.

IVBG-1A and IVBG-1B were fractionated from the Michigan plasma. The antitoxin titers of these two lots are quite similar, within the accepted range of the test procedure.

IVBG-2A and IVBG-2B were fractionated from the USAMRIID plasma. The IVBG-2B titers are lower than the IVBG-2A titers. This difference again is probably due to the method used for titering and is not due to any biological or physical differences in these two products.

The titers were reduced by 10% and reported as greater than the "adjusted" titer on the final product labels (i.e. actual titer: 224.0, labelled titer: >201). This reduction was done for all antitoxin titers for all four lots. (Compare the titers, Table XII with those printed on the final product labels, Figure 1.)

6. Immunoelectrophoresis (IEP)

Photographs of the stained immunoelectrophoresis agarose films are shown in Figures 7, 8, 9, and 10. All the final products were precipitated with anti-whole human serum. Lot number IVBG-2A was tested against anti-human total immunoglobulin and anti-human IgG in addition to anti-whole human serum.

The precipitation bands of all the final products are characteristic for intact human IgG. A minor arc appears in each final product precipitated with anti-whole human serum. This protein, determined by RID and IEP to be IgA, constitutes less than 0.2% of the total final product protein (see Table VIII and Figure 11). <u>These preparations are to be used with caution in those individuals know to have an IgA deficiency</u>.

7. Analytical and Preparative Isoelectric Focusing (IEF)

<u>Analytical</u> - Focusing is used to determine the isoelectric range and the purity of proteins. Samples of the final products are analytically focused in a pH gradient from 3.5 to 9.5. Human plasma contains four subclasses of IgG. IgG_1, 60% of the total IgG, is the predominant subclass followed by 29.5% IgG_2, 7.5% IgG_3 and 3% IgG_4 (7). The normal isoelectric range (8) for IgG_1 is pH 6.6 to 9.5. IgG_2 and IgG_3 also focus in the basic ranges from pH 6.4 to 8.3 and pH 8.2 to 9.0, respectively. The most acidic immunoglobulin, IgG_4, focuses in the range from pH 5.7 to 6.2. All final products exhibit a normal isoelectric range from pH 5.6 to pH 9.2 (Figures 12-15).

For comparison, the more acidic banding pattern indicative of fragmentation is illustrated (Figure 12) with fresh and aged commercial gamma globulins focused along with IVBG-1A. No fragmentation has occurred during fractionation and purification of the antibotulinal toxin final products.

Preparative - Preparative flatbed isoelectric focusing of IVBG-1A final product (Figure 16) shows the isoelectric range to be from pH 5.6 to 9.2. The immunoglobulins are not evenly distributed over the entire pH range but exist in varying concentrations, as determined by absorbance at 280 nm dependent upon isoelectric point. The highest concentration of immunoglobulins is found at pH 7.75. As the pH becomes more basic, the immunoglobulin concentration drops until pH 8.2 when it rises again to a second, smaller peak at pH 8.4 to 8.6.

If all the immunoglobulins were capable of neutralizing Type A botulinal toxin, we would expect to see a graph of neutralizing activity that could be superimposed upon a graph of immunoglobulin concentration. This, however, is not the case. There is toxin neutralizing antibody from pH 7.1 to 9.1 with a maximal activity at pH 8.75. Thus, the major neutralizing antibody is located in a basic isoelectric range corresponding to the isoelectric range of IgG_1, the most predominant subclass of IgG. Immunoglobulins with isoelectric points below pH 7.2 do not have measurable neutralizing activity against Type A botulinal toxin.

8. HPLEC

Size exclusion chromatography is used to separate proteins by molecular weight. Specifically, it is used to determine the amount of high molecular weight aggregates, trimers, dimers, and monomers of IgG, and low molecular weight fragments in our IVBG final products.

The HPLEC elution profiles of IVBG-1A, 1B, 2A and 2B final products (Figure 17) show one large peak (average 95.4% of the total area) at 155,000 daltons, dimers of IgG (average 3.9%) and aggregates (average 0.7%). There are no low molecular weight fragments in these final products. Thus, all final products possess the structural characteristics of human IgG, i.e. MW 155,000, and contain less than 1% aggregates and less than 1% fragments (Table XI).

9. IgG Subclasses

The subclass composition of IVBG-1A and IVBG-2B final products (Table XIII) is compared to the subclass composition of two commercial IgG preparations. Our preparations consist mainly of IgG_1 (88.9% average) and IgG_2 (10.5% average) which accounts for 99.4% of the total IgG (Tables XI and XIII). The Cutter I.V. IgG is devoid of measurable IgG_3. The Hyland ISG, prepared by cold EtOH precipitation, contains lower levels of IgG_1 and IgG_2 and contains a higher level of IgG_3 than IVBG-1A, IVBG-1B or the Cutter I.V. IgG. Subclass composition on IVBG-2A and IVBG-2B are being repeated for reasons described earlier. Upon completion, these results will be sent to USAMRIID.

10. Plasminogen

There is no detectable level of plasminogen in the IVBG final product samples (Table XIV). Plasmin contamination is undesirable, since it leads to a type of instability where functional antibody molecules are rendered inactive. However, its role in the product of adverse reactions has not been documented.

11. Prekallikrein

Although native IgG produced in this laboratory is free of prekallikrein activator (PKA) (performed by Dr. Barbara Alving, Bureau of Biologics), our native IgG does contain measurable amounts of prekallikrein (Table XIV) which can be activated to kallikrein upon the addition of activator (Factor XIIa + HMW kininogen). The most efficient removal of prekallikrein was accomplished in lots IVBG-1A, IVBG-2A and IVBG-2B. At present, there is no evidence to suggest any deleterious effects of trace amounts of prekallikrein in human I.V. gamma globulin preparations.

12. Circulatory Half-Life

Six rabbits were intravenously injected with 100 mg/kg of IVBG-1A final product. They were bled 5 minutes after injection. These samples were used to determine the initial level of circulating human IgG. Each rabbit was subsequently bled at 4, 8, 24, 48, 96, 144, 192 and 240 hours. The circulating human IgG in these samples was quantitated by the Mancini radial immunodiffusion assay. A two-

slope clearance curve was obtained from the two-phase elimination from circulation in the hours following injection of the product.

During the first 24 hour period the initial circulating level was reduced by 50% in approximately 6 hours. Following this equilibration phase the circulatory half-life was calculated to 70 hours. The half-life studies, done by USAMRIID to determine the circulating neutralizing activity are in progress. These results will be submitted to USAMRIID upon completion.

13. Anti-Complementary Activity

Anti-complementary activity--in progress. The results will be submitted to USAMRIID upon completion.

14. Limited Proteolysis by Plasmin

Samples of IVBG-1A, IVBG-1B and a commercial I.M. gamma globulin prepared by cold EtOH fractionation were subjected to proteolysis by the enzyme plasmin for 8 hours at 37°C (Figure 18). As a control, the same samples were incubated for 8 hours at 37°C without plasmin. HPLEC analysis of this experiment shows that the IVBG samples were less susceptable to plasmin degradation than the commercial IgG preparations. The IVBG-1A and IVBG-1B samples were degraded to 56% IgG monomers, 17% $F(ab')_2$, and 26% Fc fragments. In contrast, the commercial I.M. gamma globulins were degraded to 41% IgG monomers, 21% $F(ab')_2$, and 32% Fc fragments (Table XV).

II. Results

D. Shelf Life Study

The six month shelf-life study of samples stored at 37°C, room temperature (RT) 4°C and -20°C have been analyzed from all four lots. The results are as follows:

1. Neutralizing Activity

The results of the in vivo toxin neutralizing activity testing of samples stored for 6 months are shown in Table XVI.

Some decreases (and increases) in titer are shown as compared to the fresh material, but these are within the standard error of the testing procedure. Therefore, the titers have not significantly changed under any of the storage conditions.

2. Immunoelectrophoresis (IEP)

When precipitated against anti-whole human serum, the immunoelectrophoretic mobility of the samples stored for six months remains unchanged from the freshly bottled material. Only the samples stored at 37°C show any splitting of the gamma globulin precipitation arc. The other samples have remained intact. Compare Figures 7-10 (fresh) and 19-22 (stored 6 months).

3. Isoelectric Focusing (IEF)

Duplicated aliquots of each stored sample were isoelectric focused under the same conditions the fresh samples were tested. In comparison, the pI range of the fresh and stored samples are identical (5.6-9.2). See Figures 12-15 (fresh) and compare with Figures 23-26 (stored).

4. HPLEC

Sterile samples of each IVBG lot stored for 6 months at 37°C, room temperature, 4°C and -20°C are monitored by HPLEC for changes in concentration of IgG aggregates, trimers, dimers, monomers and fragments. The results of the 6 month storage study are presented in Figures 28-30 and Table XVII. There are no detectable changes in samples stored at room temperature, 4°C and -20°C. However, at 37°C there is a decrease in monomers from over 90% to between 38-73%. This reduction in monomers is reflected by increases in aggregates from less than 1% to between 1 - 16% and an increase in dimers and trimers from around 4% to between 22 - 45%. These changes are not seen earlier than 2 months and may reflect the presence of low concentrations of cathepsins in the IgG preparation. Studies are in progress to shed light on these changes.

5. Plasminogen

There is no detectable level of plasminogen in IVBG-1A, 1B, 2A and 2B final products stored for six months at -20°C, 4°C, room temperature, and 37°C (Table XIV).

6. Prekallikrein

Prekallikrein is present in IVBG-1A, 1B, 2A and 2B final products stored for six months at -20°C, 4°C, room temperature, and 37°C (Table XIV). Prekallikrein levels range from 0.4 μg/ml (2B, 6 months, 37°C) to 9.7 μg/ml (1A, 6 months, -20°C). The level of prekallikrein in samples stored at -20°C is generally higher than the fresh samples.

Discussion

The purpose of fractionating hyperimmune, human, botulinal plasma is to isolate the specific botulinal toxin neutralizing antibodies, concentrate them in a state and purity so that their biological activity will be undiminished (stable) on long-term storage (years), safe for intravenous administration and possess the circulatory half-life and distribution of native undenatured plasma (immunoglobulins).

The ion exchange method of protein separation and purification is based on the isoelectric point of the proteins. However, immunoglobulins are heterologous, complex, bifunctional proteins that differ from other plasma proteins in that they have broad isoelectric regions rather than narrow isoelectric points--a reflection of their extreme molecular heterogeneity. In hyperimmunized individuals the predominate toxin neutralizing activity will be found in the molecules of the immunoglobulin IgG class--which also makes up 70-80% of the total humoral antibodies in plasma. Therefore, any method to isolate specific neutralizing activity must isolate IgG rather than IgA or IgM.

Further, IgG is composed of four subclasses--IgG_1, IgG_2, IgG_3, and IgG_4. There is evidence that antibodies to certain groups or types of antigens can be limited to one or some of the IgG subclasses (7): antibodies to carbohydrates, dextran, or levan have been found to be exclusively IgG_2 molecules, antibodies to antigens on cell membranes are predominantly IgG_1 and IgG_3, auto-antibodies to the blood clotting factor VIII were identified as IgG_4. Antiplatelet antibodies in autoimmune thrombocytopenia purpura appear to be restricted to IgG_3. Recently, it has been reported that over 90% of the herpes virus neutralizing antibodies were also located in the IgG_3 subclass (9).

During the past 15 years, a number of observations have shown that in addition to their antibody properties, IgG molecules possess biologic activities that are controlled by their constant portion, the Fc fragment. These activities may be common to all four of the IgG subclasses or restricted to some. IgG is the only immunoglobulin class transmitted from the mother to the fetus. It seems that all four IgG subclasses are transported across the placenta. Complement activation by the classic pathway is accomplished more readily by IgG_3 and IgG_1, than by IgG_2 whereas IgG_4 does not activate at all. These differences are not seen in the alternate pathway. Also, IgG_1 and IgG_3 are the only subclasses that can induce phagocytosis of opsonized antigenic particles. IgG_3 does not react with staphylococcal protein A. Finally, and of great importance, IgG subclasses differ in their metabolic behavior: IgG_3 is more rapidly catabolized than the other three subclasses. Its biological half-life is 7 days compared to 21 days for IgG_1, IgG_2, and IgG_4.

While the IgG subclasses have broad isoelectric ranges, each subclass does have a relatively restricted region. Thus, IgG_4 has the most acidic region pI 5.7-6.2, IgG_1, the most basic, pI 6.6-9.5, IgG_2, 6.4-8.3 and IgG_3, 8.2-9.0.

The relative concentration is of great practical importance since IgG_1 represents 60% of total IgG, IgG_2 30%, IgG_3 5% and IgG_4 2.0%.

Ideally, the most effective I.V. human, hyperimmune, antibotulinal IgG should contain only the neutralizing IgG molecules. However, such a preparation could only be prepared by an affinity method involving immobilized botulinal toxins which are reacted with hyperimmune plasma and the specific antitoxin molecules eluted and concentrated. We have developed methods of isolating the predominant neutralizing

activity which is reflected by a high specific toxin neutralizing activity. To demonstrate this fact we have performed preparative isoelectric focusing of hyperimmune, human plasma eluting proteins from each segment and determining the toxin neutralizing activity to botulinal toxin A. These studies show that the major neutralizing activity was located in the most basic pI region which also corresponds to the pI region of the IgG_1 subclass. Therefore, the ion exchange method should isolate and concentrate this relatively high pI region protein. This is documented in Figure 16 which demonstrates that for the IVBG-1A final product, the region of highest toxin neutralizing activity is comparable to the highest pI region of the hyperimmune plasma where the predominate neutralizing activity was located.

While IgG_3 is removed in our method by SiO_2 treatment, the toxin neutralizing activity is not appreciably affected. This loss of the subclass should have little effect since IgG_3 has the shortest half-life, 7 days vs. 21 days for IgG_1, and is lowest in relative concentrations, 5% vs. 60% of IgG_1.

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FRACTIONATION FLOW SHEET 1

Plasma Pool

↓

Addition of Silicon Dioxide, SiO_2

↓

Removal of SiO_2 By Centrifugation

↓

Sterilization By 0.2 Micron Filtration

↓

Concentration and Buffer Exchange

↓

Anion Exchange Chromatography

↓

Concentration and Buffer Exchange

↓

Sterilization By 0.2 Micron Filtration

↓

Bottling

Fractionation Flow Sheet #2

(IVBG-1A)

	Liters	Protein† mg/ml	Protein grams	IgG grams
Plasma Pool	230.0	48.7	11,190	1325
↓				
SiO_2 Adsorption 28 gm/l				
↓				
SiO_2 Removal: Low-speed spin, Sorvall RC3, 4°C, 6975 *g*.				
↓				
SiO_2 Removal: High-speed spin, Beckman J2-21, Continuous-flow, 4°C, 32,260 *g*.				
↓				
0.2 Micron Sterile Filtration	302.3	27.0	8,149	1026
↓				
Concentration and Diafiltration				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 32,260 *g*.	96.89	79.8	7,734	974
↓				
QAE 1: A-50 Sephadex, pH 6.6, Cond. 5.85mS at 21°C				
↓				
Concentration				
↓				
QAE 2: A-50 Sephadex, pH 6.6, Cond. 5.85mS at 21°C	52.0	14.4	747.0	735.8
↓				
Concentration and Diafiltration				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 39,260 *g*.	6.039	103.1	622.62	613.2
↓				
Pre-filtration 0.2 Micron				
↓				
Sterile Filtration/Bottling	5.92	103.1	609.76	600.9

†The protein solutions are stored in two or more containers at each stage. The protein concentration shown is an average of the biuret determination for each container.

Fractionation Flow Sheet #3
(IVBG-1B)

	Liters	Protein† mg/ml	Protein grams	IgG grams
Plasma Pool	233.4	51.3	11,985	1211
↓				
SiO_2 Adsorption 30 gm/l				
↓				
SiO_2 Removal: Low-speed spin, Sorvall RC3, 4°C, 6975 *g*.				
↓				
SiO_2 Removal: High-speed spin, Beckman J2-21, Continuous-flow, 4°C, 32,260 *g*.				
↓				
0.2 Micron Sterile Filtration	209.0	39.5	8,244	960
↓				
Concentration and Diafiltration				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 32,260 *g*.	98.43	79.3	7,805	909
↓				
QAE 1: A-50 Sephadex, pH 6.6, Cond. 5.85mS at 21°C	112.0	6.1	683.0	687
↓				
Concentration and Diafiltration				
↓				
Addition of IVBG-1A Remnants				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 39,260 *g*.	6.75	108.0	729.0	732.0
↓				
Pre-filtration 0.2 Micron				
↓				
Sterile Filtration/Bottling	6.74	108.0	727.92	731.9
↓				
Second Bottling	5.69	108.0	614.52	617.9

†The protein solutions are stored in two or more containers at each stage. The protein concentration shown is an average of the biuret determination for each container.

Fractionation Flow Sheet #4

(IVBG-2A)

	Liters	Protein† mg/ml	Protein grams	IgG grams
Plasma Pool	234.3	50.8	11,903	1361
↓				
SiO_2 Adsorption 30 gm/l				
↓				
SiO_2 Removal: Low-speed spin, Sorvall RC3, 4°C, 6975 *g*.				
↓				
SiO_2 Removal: High-speed spin, Beckman J2-21, Continuous-flow, 4°C, 32,260 *g*.				
↓				
0.2 Micron Sterile Filtration	239.0	34.4	8,225.0	951.4
↓				
Concentration and Diafiltration				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 32,260 *g*.	98.60	80.1	7,902.0	914.0
↓				
QAE 1: A-50 Sephadex, pH 6.6 Cond. 5.85mS at 21°C	104.0	6.4	661.0	696.9
↓				
Concentration and Diafiltration				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 39,260 *g*.	5.92	105.0	621.60	655.3
↓				
Pre-filtration 0.2 Micron				
↓				
Sterile Filtration/Bottling	5.93	105.0	622.65	656.0

†The protein solutions are stored in two or more containers at each stage. The protein concentration shown is an average of the biuret determination for each container.

Fractionation Flow Sheet #5

(IVBG-2B)

	Liters	Protein† mg/ml	Protein grams	IgG grams
Plasma Pool	240.0	49.4	11,844	1313
↓				
SiO_2 Adsorption 30 gm/l				
↓				
SiO_2 Removal: Low-speed spin, Sorvall RC3, 4°C, 6975 *g*.				
↓				
SiO_2 Removal: High-speed spin, Beckman J2-21, Continuous-flow, 4°C, 32,260 *g*.				
↓				
0.2 Micron Sterile Filtration	232.2	32.7	7,596	728
↓				
Concentration and Diafiltration				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 32,260 *g*.	89.97	80.0	7,199.0	690.0
↓				
QAE 1: A-50 Sephadex, pH 6.6, Cond. 5.85mS at 21°C	96.0	5.3	506.00	499.0
↓				
Concentration and Diafiltration				
↓				
Warm-up High-speed Spin: Beckman J2-21, Continuous-flow, 20°C, 39,260 *g*.	4.75	103.0	489.25	480.7
↓				
Pre-filtration 0.2 Micron				
↓				
Sterile Filtration/Bottling	4.41	103.0	452.47	448.0

†The protein solutions are stored in two or more containers at each stage. The protein concentration shown is an average of the biuret determination for each container.

Table I

FILTERS USED IN PRODUCTION OF IVBG LOTS 1A, 1B, 2A and 2B

		IVBG Lot			
Manufacturer	Filter Part No.*	1A	1B	2A	2B
PALL	AB1NR7P	X	X	X	X
PALL	AB1NA3P	X			
PALL	AB2NA8P	X			
PALL	DFA3001ARP	X	X	X	
PALL	AB2NA7P		X		X
PALL	FLF6001ARP		X	X	X
PALL	AB1NA7P		X		X
PALL	SLK7001ARP			X	
PALL	DFA3001NRP				X
PALL	SLK7001NRP				X
GELMAN	12645			X	
GELMAN	12122			X	

Pall Trinity Micro Corporation
Cortland, NY 13045

Gelman Sciences, Inc.
600 South Wagner Road
Ann Arbor, MI 48106

*All filters are manufactured in accordance with Current Good Manufacturing Practices (C.G.M.P.) guidelines and are non-fiber releasing.

Table II

DISTRIBUTION OF PLASMA UNITS* IN IVBG LOTS

	IVBG-1A	IVBG-1B	IVBG-2A	IVBG-2B
Source:	1st half of 7/15/81 shipment from Michigan	2nd half of 7/15/81 shipment from Michigan	1st half of 8/12/81 shipment from USAMRIID	2nd half of 8/12/81 shipment from USAMRIID
Number of Units:	683	682	699	695
Pool Volume (liters):	230.0	233.4	234.3	240.0

*A list of unit donor numbers for each lot is contained in Appendix

Table III

REMOVAL OF LIPOPROTEIN DURING FRACTIONATION

Sample	Triglyceride mg/ml	Cholesterol mg/ml	(HDL) αLipoprotein mg/ml	(LDL) βLipoprotein mg/ml
IVBG-1A:				
Plasma Pool	1.63	1.38	1.29	2.00
Pre QAE	0.13	ND	ND	ND
Final Product	ND	ND	ND	ND
IVBG-1B:				
Plasma Pool	2.16	1.61	0.54	2.73
Pre QAE	ND	ND	ND	ND
Final Product	ND	ND	ND	ND
IVBG-2A:				
Plasma Pool	2.21	1.65	0.55	3.01
Pre QAE	ND	ND	ND	ND
Final Product	ND	ND	ND	ND
IVBG-2B:				
Plasma Pool	2.07	1.63	0.56	3.06
Pre QAE	ND	ND	ND	ND
Final Product	ND	ND	ND	ND

ND: Not Detectable

Table IV

PLASMINOGEN AND PREKALLIKREIN LEVELS

DURING FRACTIONATION

Sample	Plasminogen			Prekallikrein		
	CTA U/ml	CTA U/mg	Total Units	µg/ml	µg/mg	Total Grams
IVBG-1A:						
Plasma Pool	*3.12	0.064	716,000	11.2	0.23	2.6
Pre QAE	ND	ND	ND	2.0	0.03	0.2
Final Product	ND	ND	ND	1.8	0.02	0.01
IVBG-1B:						
Plasma Pool	3.50	0.068	815,000	4.6	0.09	1.1
Pre QAE	ND	ND	ND	1.5	0.02	0.2
Final Product	ND	ND	ND	3.8	0.04	0.02
IVBG-2A:						
Plasma Pool	3.06	0.060	714,000	8.7	0.17	2.0
Pre QAE	ND	ND	ND	1.6	0.02	0.2
Final Product	ND	ND	ND	2.2	0.02	0.01
IVBG-2B:						
Plasma Pool	3.52	0.071	841,000	4.1	0.08	0.9
Pre QAE	ND	ND	ND	0.9	0.01	0.1
Final Product	ND	ND	ND	0.5	<0.01	<0.01

*ND: Not Detectable

Lowest Detection Level: Plasminogen - 0.01 CTA U/ml

Prekallikrein - 0.10 µg/ml

Table V

THE EFFECT OF SiO_2 AND ANION EXCHANGE ON IgG SUBCLASSES** DURING FRACTIONATION

Sample	*Total IgG Grams	*Total IgG %	IgG_1 % of Total IgG	IgG_2 % of Total IgG	IgG_3 % of Total IgG	IgG_4 % of Total IgG
IVBG-1A:						
Plasma Pool	1325	100	100	100	100	100
Pre QAE	974	74	81	72	0.4	98
Final Product	672	51	89.5	9.8	0.3	0.4
IVBG-1B:						
Plasma Pool	1211	100	100	100	100	100
Pre QAE	909	75	96	43	0.5	84
Final Product	659	54	88.2	11.1	0.1	0.6
IVBG-2A:††						
Plasma Pool	1361	100	100	100	100	100
Pre QAE	914	67	97	38	1.4	78
Final Product	656	48	67.3	22	4.3	6.3
IVBG-2B: ††						
Plasma Pool	1313	100	100	100	100	100
Pre QAE	690	53	63	22	10	59
Final Product	452	34	79	16.3	2.6	2.1

* IgG Determined by Radial Immunodiffusion Assay.

** Subclasses Determined by RadioImmuno Assay.

†† IVBG-2A and IVBG-2B have been resubmitted for repeat testing.

Table VI

QAE A-50 ANION EXCHANGE CHROMATOGRAPHY*

	IVBG-1A	IVBG-1B	IVBG-2A	IVBG-2B
Protein Applied:				
Volume (liters)	96.89	98.43	98.60	89.97
** Total Protein Concentration (mg/ml)	79.8	79.3	80.2	80.1
Total Protein (grams)	7734	7805	7902	7199
Ratio of Total Protein to QAE (gm/gm)	1.45	1.48	1.50	1.37
***IgG Concentration (mg/ml)	10.05	9.23	9.27	7.67
IgG (grams)	974	909	914	690
Protein Collected:				
IgG (grams)	747.0	682.5	661.0	506.0
IgG Yield vs. Total Protein Applied	9.66%	8.74%	8.36%	7.03%
IgG Yield vs. IgG grams Applied	76.7%	75.1%	72.3%	73.3%

*pH 6.6 conductivity 5.85 mS at 21°C

**The protein solutions are stored in two or more containers at each stage. The protein concentration shown is an average of the biuret determination for each container.

***IgG concentration determined by Radial Immunodiffusion (R.I.D.) Assay.

Table VII

COMPARISON OF SOME PLASMA PROTEINS*

DURING FRACTIONATION**

Sample	Protein*** mg/ml	Transferrin mg/ml	α_2Macroglobulin mg/ml	Hemopexin mg/ml	Albumin mg/ml
IVBG-1A:					
Plasma Pool	48.7	1.88	0.84	0.72	27.7
Pre QAE	79.8	0.93	1.70	0.52	60.0
Final Product	1[illegible]	<0.02	ND†	ND	ND
IVBG-1B:					
Plasma Pool	51.3	2.02	0.80	0.78	31.8
Pre QAE	79.3	4.15	1.73	0.49	61.4
Final Product	108.0	<0.02	ND	ND	ND
IVBG-2A:					
Plasma Pool	50.8	2.46	0.90	0.77	33.5
Pre QAE	80.1	4.73	2.00	0.40	62.8
Final Product	105.0	<0.02	ND	ND	ND
IVBG-2B:					
Plasma Pool	49.4	2.14	0.88	0.79	30.3
Pre QAE	80.0	4.68	1.77	0.20	62.8
Final Product	103.0	<0.02	ND	ND	ND

*Values determined by Radial Immunodiffusion (R.I.D.) Assay.

**The Pre-QAE plasma has been concentrated to approximately 80 mg protein/ml.

***The protein solutions are stored in two or more containers at each stage. The protein concentration shown is an average of the biuret determination for each container.

†ND: Not Detectable

Table VIII

COMPARISON OF VARIOUS IMMUNOGLOBULINS*

DURING FRACTIONATION**

Sample	Protein† mg/ml	IgG mg/ml	IgA mg/ml	IgM mg/ml
IVBG-1A:				
Plasma Pool	48.7	5.76	1.06	0.84
Pre QAE	79.8	10.05	2.42	1.11
Final Product	103.1	101.5	<0.02††	ND
IVBG-1B:				
Plasma Pool	51.3	5.19	1.19	0.74
Pre QAE	79.3	9.23	2.33	0.76
Final Product	108.0	108.6	<0.02††	ND
IVBG-2A:				
Plasma Pool	50.8	5.81	1.32	0.94
Pre QAE	80.1	9.27	2.72	0.76
Final Product	105.0	110.7	<0.02††	ND
IVBG-2B:				
Plasma Pool	49.4	5.47	1.21	0.80
Pre QAE	80.0	7.67	2.53	0.60
Final Product	103.0	101.6	<0.02††	ND
Average:				
Plasma Pool	50.1	5.55	1.20	0.83
Pre QAE	79.9	9.06	2.50	0.81
Final Product	104.8	105.6	<0.02††	ND

*Values determined by Radial Immunodiffusion (R.I.D.) Assay.

**The Pre-QAE plasma has been concentrated to 80 mg protein/ml.

†The protein solutions are stored in two or more containers at each stage. The protein concentration shown is an average of the biuret determination for each container.

††Represents trace amounts of IgA. Caution should be taken when administered to persons known to be deficient in IgA.

Table IX

FRACTIONATION YIELDS

Sample	Total Protein* Vol. (ℓ)	Grams	% Yield	IgG** Grams	% Yield
IVBG-1A:					
Plasma Pool	230.0	11,190	100.0	1,325	100.0
Pre QAE	96.9	7,734	69.1	974	73.5
Final Product Bottled	*** 5.92	609.8	5.46	601	45.4
IVBG-1B:					
Plasma Pool	233.4	11,985	100.0	1,211	100.0
Pre QAE	98.4	7,805	65.1	909	75.1
Final Product Bottled	*** 6.74	727.9	6.07	732	60.4
2nd Bottling	5.69	614.5	5.13	618	51.0
IVBG-2A:					
Plasma Pool	234.3	11,903	100.0	1,361	100.0
Pre QAE	98.6	7,902	66.4	914	67.2
Final Product Bottled	5.93	622.7	5.23	656	48.2
IVBG-2B:					
Plasma Pool	240.0	11,844	100.0	1,313	100.0
Pre QAE	90.0	7,199	60.8	690	52.6
Final Product Bottled	4.41	454.2	3.83	448	34.1
Totals:					
Plasma Pool	937.7	46,922	100.0	5,210	100.0
Pre QAE	383.9	30,640	65.4	3,487	67.1
Final Product Bottled	21.95	2,301.2	4.91	2,323	44.6

* Total Protein Determined By Biuret.
** IgG Levels Determined By Radial Immunodiffusion Assay.
*** Grams of Final Product Bottled. An Additional 73 Grams of IgG Not Bottled with IVBG-1A was Bottled with and is Included in the IVBG-1B Totals.

Table X

FINAL PRODUCT SUMMARY I

	IVBG-1A	IVBG-1B	IVBG-1B (Rebottled)	IVBG-2A	IVBG-2B
Date Bottled:	10/02/81	5/07/82	12/09/82	7/30/82	8/27/82
Expiration Date:	Oct. 1991	May 1992	May 1992	July 1992	Aug. 1992
Pre Bottle Volume (liters):	6.039	6.75	5.69	5.92	4.75
Prefilter:	N2730206*	KO210038*	NRPM2490299*	N4840290*	M2500144*
Final Filter (.2μ) Type:	ARP 0270186** ARP 0270015**	NRP NO180149**	NRP RO970122**	ARP RO070109**	NRP N6890194**
Protein Concentration (mg/ml):	103.1	108.0	108.0	105.0	103.0
Total # Vials Bottled:	592	674	569	593	441
# Vials for Testing:	56	56	34	51	52
# Vials to Ship:	537	618	535	536	389
Grams per Vial:	1.03	1.08	1.08	1.05	1.03
Total Grams Bottled:	609.76	727.92	614.52	622.65	454.23
Total Grams to Ship:	553.11	667.44	577.8	562.8	400.67

*Pall, Sealkleen

**Pall, DFA

Table XI

FINAL PRODUCT SUMMARY II, TESTING

	IVBG-1A	IVBG-1B (rebottled)	IVBG-2A	IVBG-2B
Conductivity (mS):	11.45 at 21°C	10.65 at 20°C	11.40 at 22°C	11.45 at 22°C
pH:	7.42	6.78	6.77	6.83
Sterility:	Sterile*	Sterile*	Sterile*	Sterile*
Pyrogenicity:	Non-pyrogenic	Non-pyrogenic	Non-pyrogenic	Non-pyrogenic
Safety:	Passed	Passed	Passed	Passed
Hepatitis -				
Antigen:	Negative	Negative	Negative	Negative
Antibody:	Positive	Positive	Positive	Positive
Anti-C' Activity	In Progress	In Progress	In Progress	In Progress
IgG Subclass Composition (% of total) -				
IgG_1	89.5	88.2	Resubmitted	Resubmitted
IgG_2	9.8	11.1	Resubmitted	Resubmitted
IgG_3	0.3	0.1	Resubmitted	Resubmitted
IgG_4	0.4	0.6	Resubmitted	Resubmitted
HPLEC -				
(% of) Dimers:	4.6	4.7	3.5	2.7
(% Of) Aggregates:	0.7	0.9	0.7	0.7
(% of) Monomers:	94.7	94.4	95.8	96.6

*Contains No Preservatives

Table XII

ANTI-BOTULINAL TOXIN TITERS

	Neutralizing Activity IU/ml				Specific Activity IU/mg			
Toxin Type	IVBG-1A	IVBG-1B	IVBG-2A	IVBG-2B	IVBG-1A	IVBG-1B	IVBG-2A	IVBG-2B
A	100.8	224.0	280.0	77.0	0.978	2.074	2.667	.748
B	9.6	10.2	34.6	12.3	0.093	0.094	.183	.119
C	112.9	80.0	40.4	45.8	1.100	0.741	.385	.445
D	181.6	169.0	174.0	54.0	1.761	1.565	1.657	.524
E	72.6	75.0	87.4	23.4	0.704	0.694	.832	.227

$$\text{Specific Activity} = \frac{\text{IU/ml}}{\text{mg/ml}}$$

Table XIII

PERCENTAGES OF IgG SUBCLASSES FROM COMMERCIAL GAMMA GLOBULIN AND FROM IVBG FINAL PRODUCTS

Sample	Protein mg/ml	% of Total IgG			
		IgG_1	IgG_2	IgG_3	IgG_4
IVBG-1A Final Product	103.1	89.5	9.8	0.3	0.4
IVBG-1B Final Product	108.0	88.2	11.1	0.1	0.6
IVBG-2A Final Product††	105.0	67.3	22.0	4.3	6.3
IVBG-2B Final Product††	103.0	79.0	16.3	2.6	2.1
Cutter I.V. IgG*	59.7	84.6	12.3	ND†	3.1
Hyland ISG	157.5	75.8	4.2	19.2	0.7

*Cutter Gamimmune, Lot #C5755, Reduced and Alkylated, Exp. 31, October 1982.

**Hyland Immune Serum Globulin, Lot #2703 T 001 FA, Exp. 31, August 1984

†ND: Not Detectable

††IVBG-2A and IVBG-2B have been resubmitted for repeat testing.

Table XIV

PLASMINOGEN AND PREKALLIKREIN LEVELS OF FINAL PRODUCTS

FRESH AND 6 MONTH SHELF LIFE SAMPLES

	Plasminogen (CTA U/ml)				Prekallikrein (mg/ml)			
Storage Conditions	1A	1B	2A	2B	1A	1B	2A	2B
0 Months/Fresh	*ND	ND	ND	ND	1.8	3.8	2.2	0.5
6 Months/-20°C	ND	ND	ND	ND	9.7	6.6	2.7	1.5
6 Months/4°C	ND	ND	ND	ND	8.2	3.2	8.0	2.7
6 Months/Room Temperature	ND	ND	ND	ND	4.5	3.8	6.1	2.2
6 Months/37°C	ND	ND	ND	ND	0.6	3.8	7.7	0.4

*ND: Not Detectable

Lowest Detection Level: Plasminogen - 0.01 CTA U/ml
Prekallikrein - 0.10 µg/ml

Table XV

LIMITED PROTEOLYSIS OF IVBG-1A AND IVBG-1B WITH PLASMIN

Sample	Percent of Total Area					
	Aggregates >500 K	IgG Dimers and Trimers	IgG Monomers ∿155 K	$F(ab')_2$ ∿95 K	Fc (∿31 K)	Low MW Fragments
IVBG-1A						
With Plasmin	0	1.4	56.5	17.8	24.3	<1
Without Plasmin	0	3.4	96.6	0	0	0
IVBG-1B						
With Plasmin	0	1.1	55.2	16.4	27.3	<1
Without Plasmin	0	1.8	98.2	0	0	0
Commercial I.M. ISG						
With Plasmin	0.8	5.0	41.3	20.7	32.2	<1
Without Plasmin	1.5	29.1	69.4	0	0	0

Table XVI

6 MONTH SHELF LIFE SAMPLES:

TYPE A, B, C, D AND E NEUTRALIZING TITERS ON IVBG FINAL PRODUCTS

	Storage Conditions	Neutralizing Activity (IU/ml) Type A	Type B	Type C	Type D	Type E
IVBG-1A	Fresh - 0 months	219.0	11.4	106.8	121.8	92.1
	-20°C - 6 months	320.0	10.0	358.0	129.0	144.0
	4°C - 6 months	201.6	16.0	285.0	325.0	113.5
	22°C - 6 months	204.0	12.2	89.6	325.0	51.2
	37°C - 6 months	80.6	6.3	89.6	102.0	57.6
IVBG-1B	Fresh - 0 months	224.0	10.2	80.0	169.0	75.0
	-20°C - 6 months	242.0	32.0	NT*	NT	NT
	4°C - 6 months	NT	25.0	NT	NT	NT
	22°C - 6 months	237.0	32.0	NT	NT	NT
	37°C - 6 months	242.0	25.0	NT	NT	NT
IVBG-2A	Fresh - 0 months	280.0	34.6	40.4	17.4	87.4
	-20°C - 6 months	NT	22.0	NT	NT	NT
	4°C - 6 months	NT	33.0	NT	NT	NT
	22°C - 6 months	NT	33.0	NT	NT	NT
	37°C - 6 months	NT	22.0	NT	NT	NT
IVBG-2B	Fresh - 0 months	77.0	12.3	NT	NT	NT
	-20°C - 6 months	89.0	12.8	NT	NT	NT
	4°C - 6 months	67.0	12.8	NT	NT	NT
	22°C - 6 months	45.0	12.8	NT	NT	NT
	37°C - 6 months	45.0	12.8	NT	NT	NT

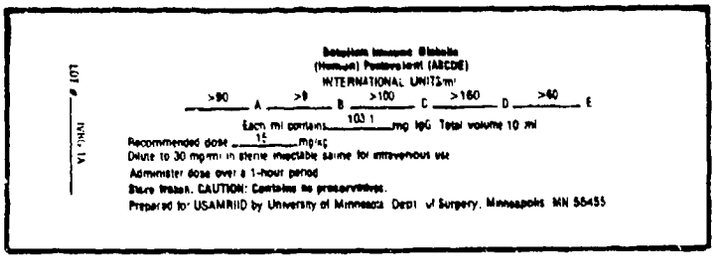
*NT--Not Tested

Table XVII

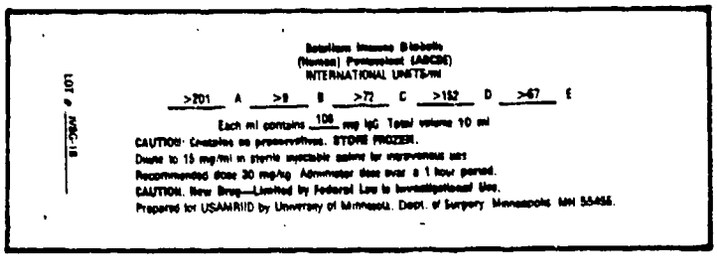
SIX MONTH STABILITY OF IVBG LOTS: EFFECT OF STORAGE TEMPERATURE ON AGGREGATE FORMATION

Final Product Stored 6 Months	Percent of Total Area			
	Aggregates	IgG Dimers and Trimers	IgG Monomers	Fragments
IVBG-1A				
-20°C	0.8	6.0	93.1	0
4°C	0.4	4.9	94.7	0
22°C	0.5	7.5	91.9	0
37°C	8.9	36.9	52.6	1.6
IVBG-1B				
-20°C	0.5	4.1	95.4	0
4°C	0.5	2.5	97.0	0
22°C	0.3	5.8	93.9	0
37°C	15.7	44.6	38.0	1.7
IVBG-2A				
-20°C	0.6	5.1	94.3	0
4°C	0.3	4.9	94.8	0
22°C	0.4	4.5	95.1	0
37°C	3.5	28.6	65.8	2.1
IVBG-2B				
-20°C	0.4	4.3	95.3	0
4°C	0.6	4.4	95.0	0
22°C	0.1	3.0	96.9	0
37°C	1.7	22.1	72.6	3.5

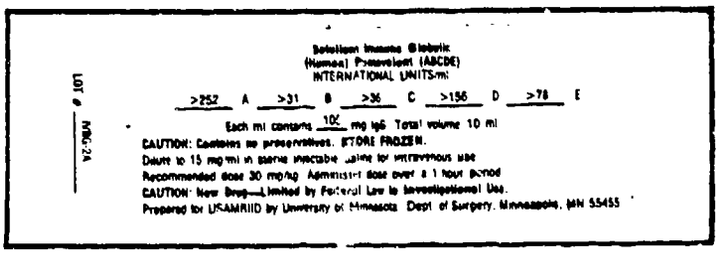
Custom Designed Final Product Labels



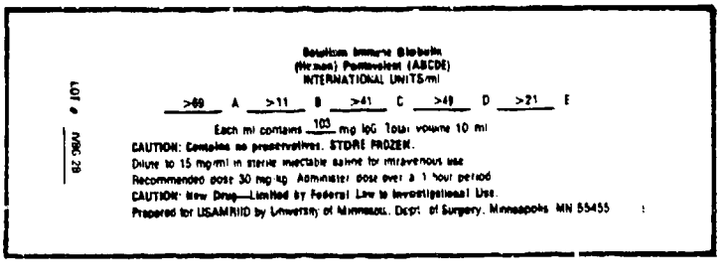
IVBG-1A



IVBG-1B



IVBG-2A



IVBG-2B

Protein and IgG Yields During Fractionation

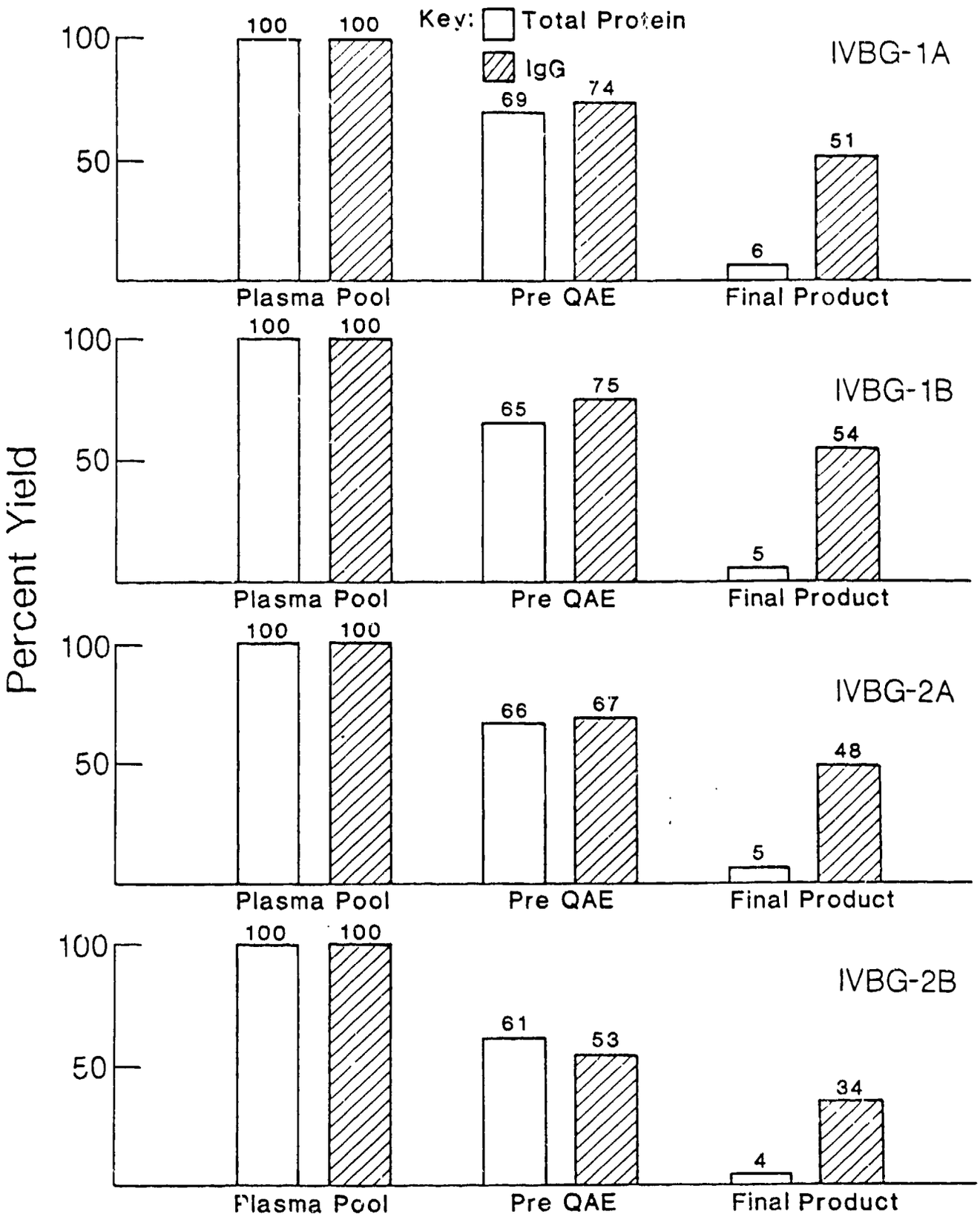
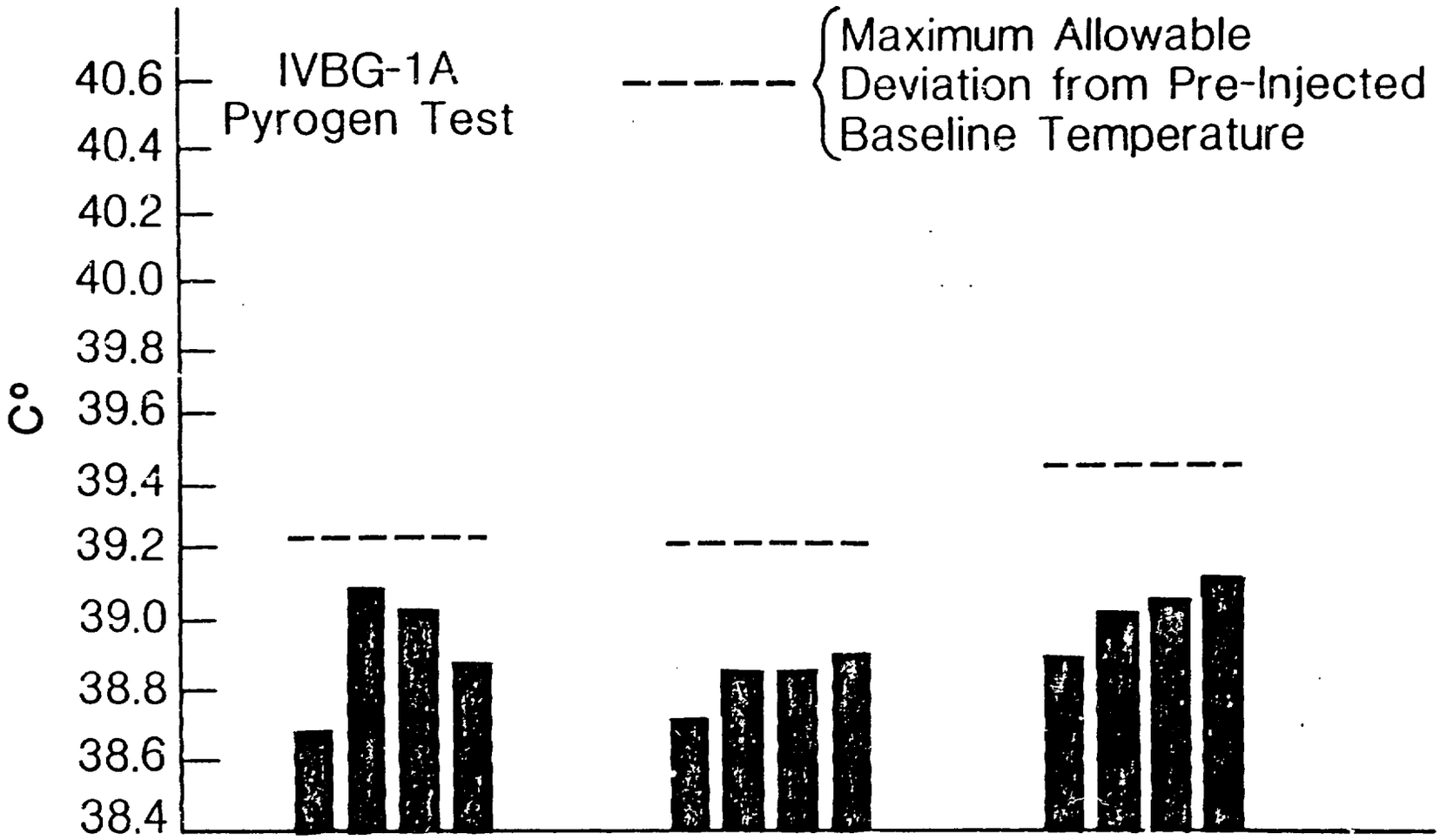


Figure 3 87

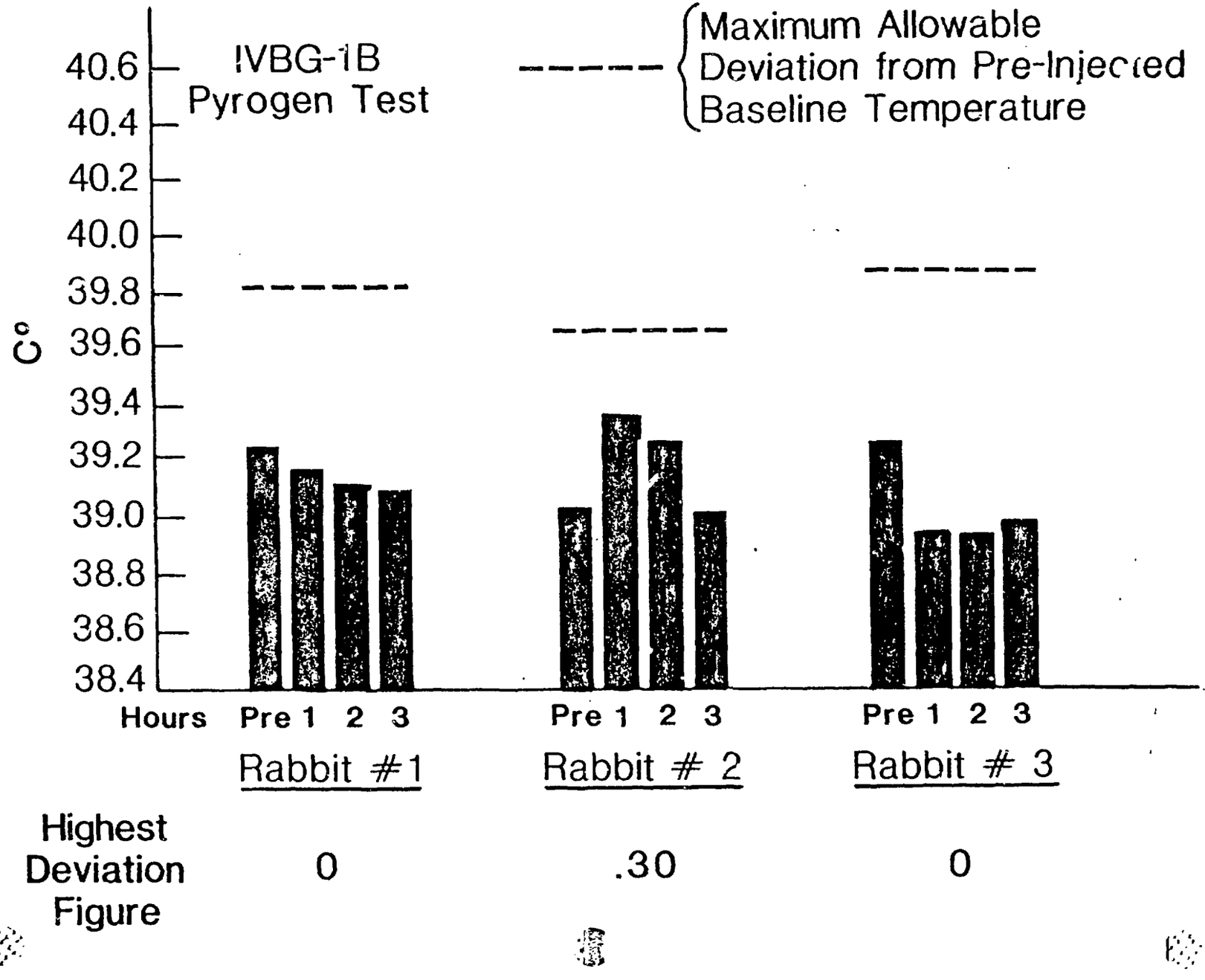
Pyrogen Testing of IVBG-1A



	Rabbit #1	Rabbit # 2	Rabbit # 3
Highest Deviation Figure	.45	.25	.25

Figure 4

Pyrogen Testing of IVBG-1B

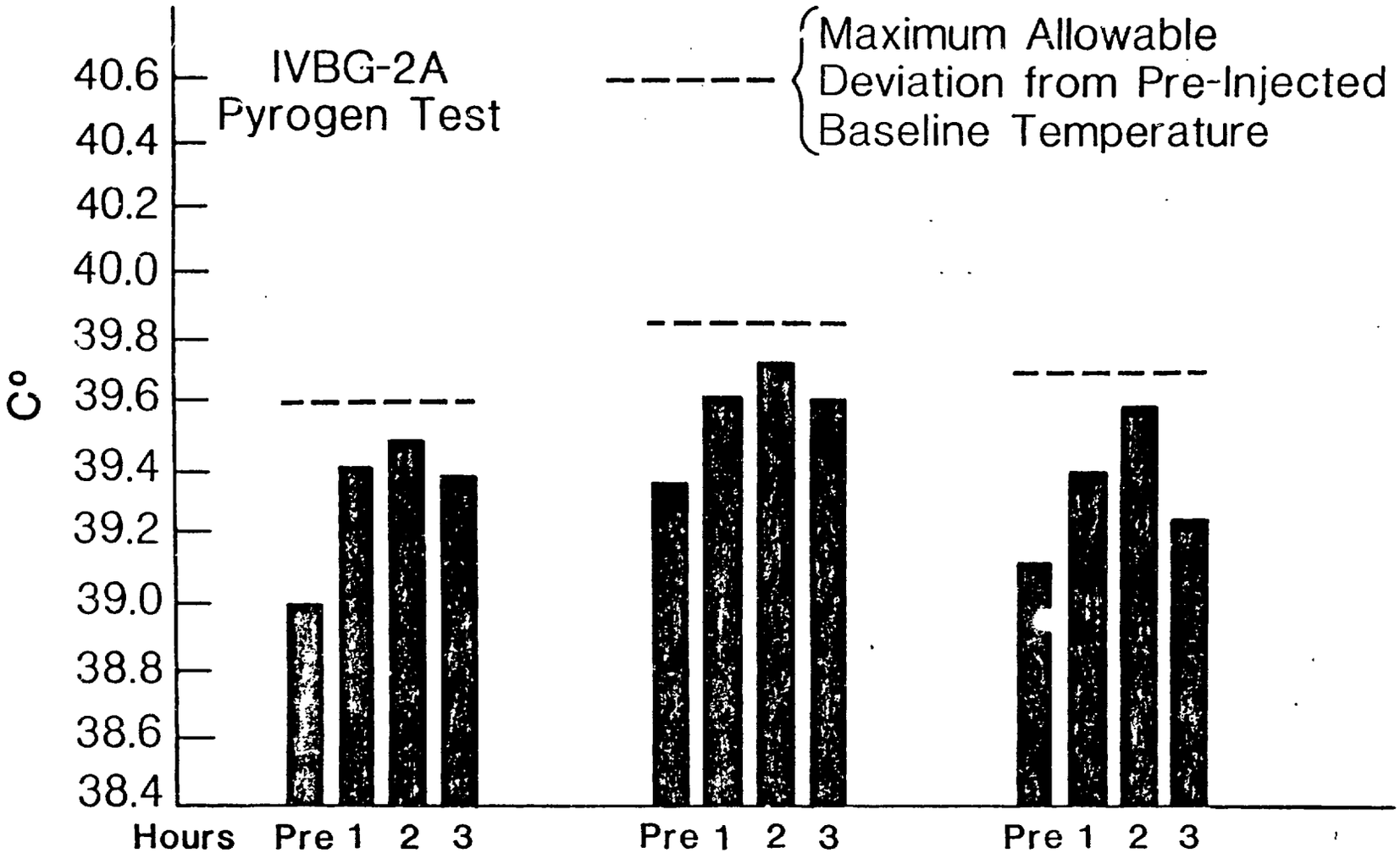


	Rabbit #1	Rabbit # 2	Rabbit # 3
Highest Deviation Figure	0	.30	0

Figure 5

89

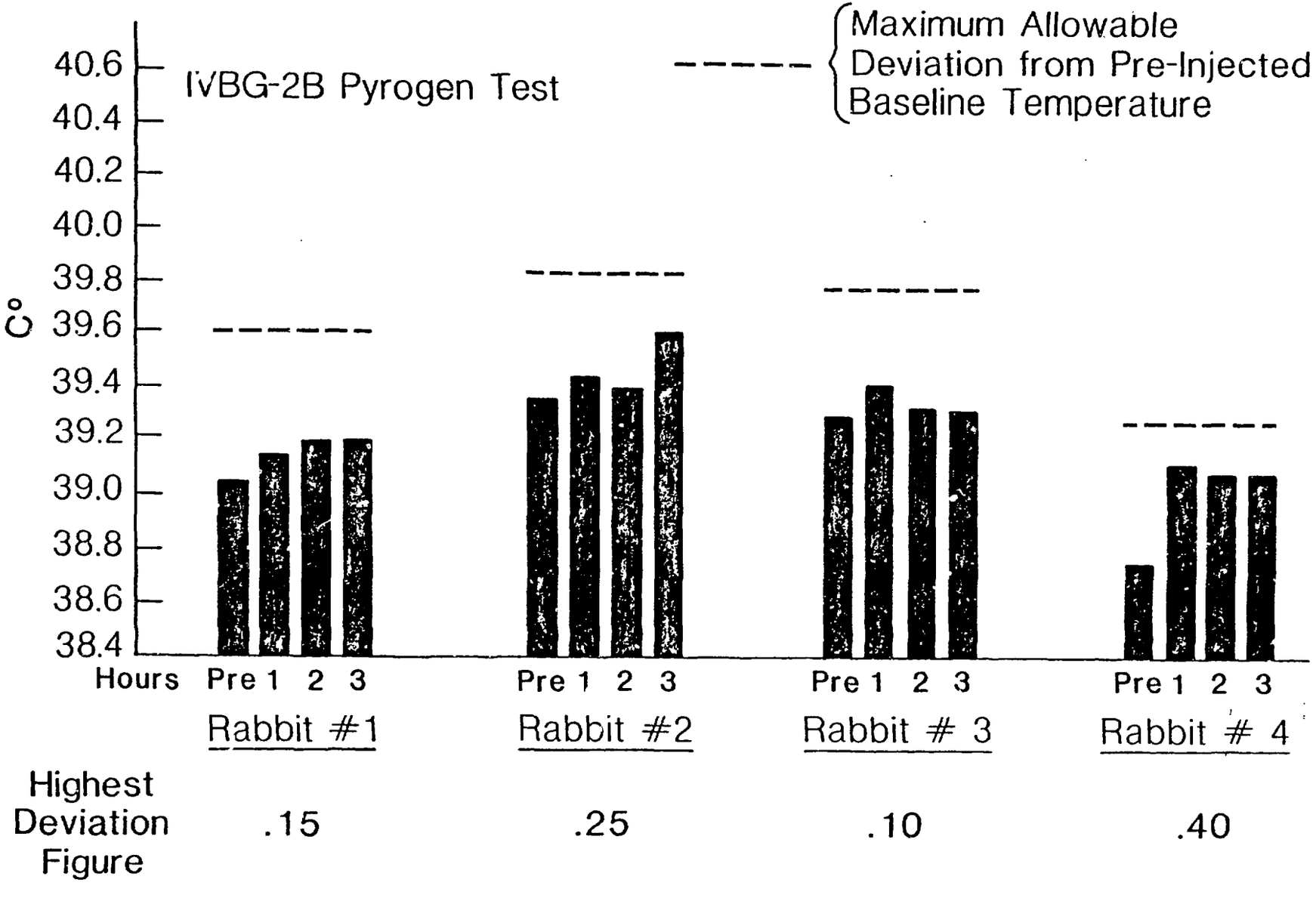
Pyrogen Testing of IVBG-2A



	Rabbit #1	Rabbit # 2	Rabbit # 3
Highest Deviation Figure	.50	.40	.50

Figure 6

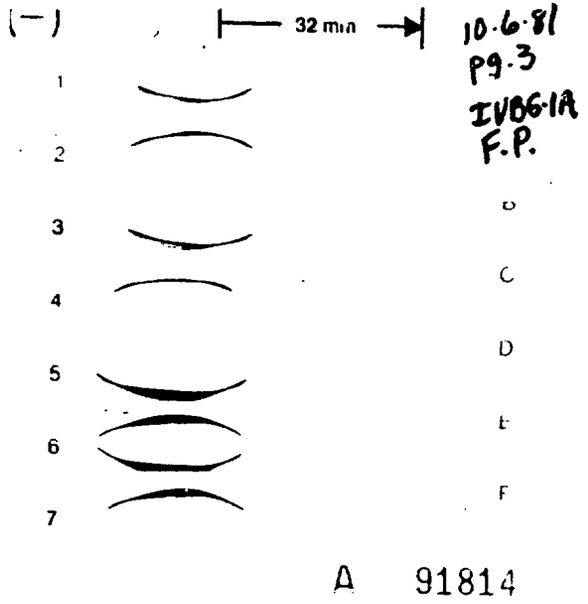
Pyrogen Testing of IVBG-2B



	Rabbit #1	Rabbit #2	Rabbit # 3	Rabbit # 4
Highest Deviation Figure	.15	.25	.10	.40

Figure 7 91

Immunoelectrophoresis of IVBG-1A Final Product

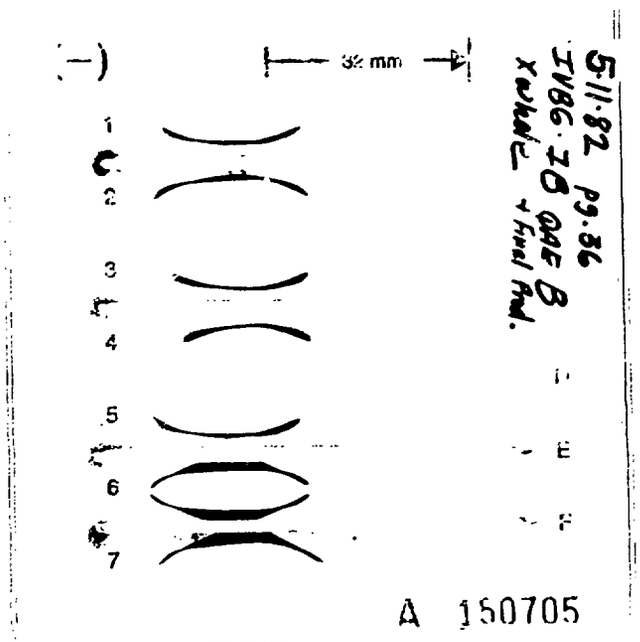


			mg/ml
1	IVBG-1A Post QAE C Elution	ℓ.17	10.0
2	↓	ℓ.18	10.0
3	↓	ℓ.19	10.0
4	Final Product		10.0
5	Pre QAE C Pool		50.8
6	Final Product		51.0
7	Final Product		103.1

Samples Electrophoresed for 75 Minutes at 80 Volts
Antisera: Kallestad, Rabbit Anti Whole Human, Lot #301L011

Figure 8

Immunoelectrophoresis of IVBG-1B Final Product

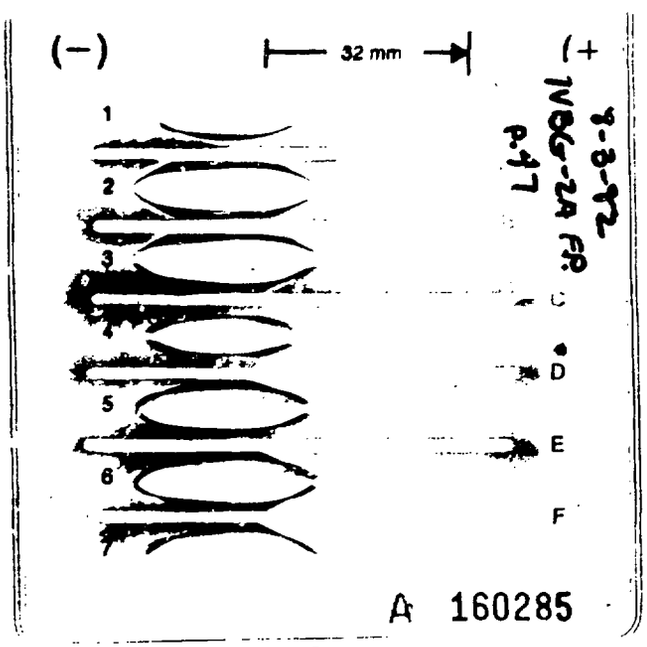


			mg/ml
1	IVBG-1B Post QAE B	*l*. 56	10.0
2	Post QAE B	*l*. 60	10.0
3	Post QAE B	*l*. 64	4.6
4	Post QAE B	*l*. 68	3.4
5	IVBG -1B Final Product		10.0
6	Final Product		50.0
7	Final Product		108.0

Samples Electrophoresed for 90 Minutes at 80 Volts
Antisera: Kallestad, Rabbit Anti Whole Human, Lot # 301L011

Figure 9

Immunoelectrophoresis of IVBG-2A Final Product



		mg/ml
1	IVBG-2A Final Product	10.0
2 *		60.0
3		105.0
4 *		10.0
5 ▪		60.0
6 ▪		105.0
7 •		105.0

Samples Electrophoresed for 90 Minutes at 80 Volts

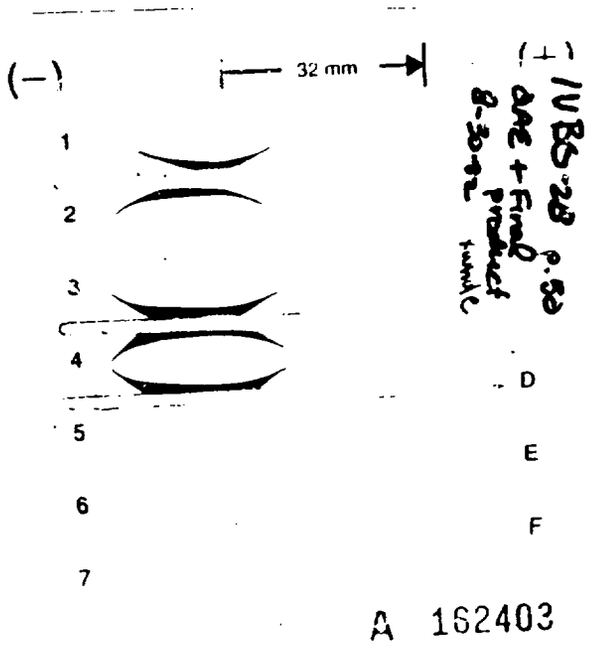
Antisera: * Gibco, Rabbit Anti Whole Human, Lot #30N911

▪ Kallestad, Goat Anti Total Immunoglobin, Human, Lot #213M01B

• Kallestad, Rabbit Anti IgG Human, Lot #204M05F

Figure 10

Immunoelectrophoresis of IVBG-2B Final Product

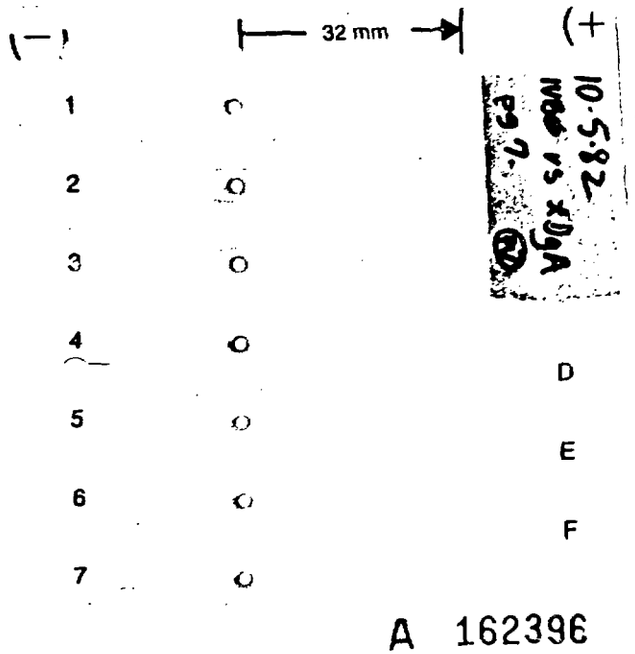


1	IVBG-2B Post QAE B 1.60	10.0
2	Final Product	10.0
3	Final Product	60.0
4	Final Product	103.0
5		
6		
7		

Samples Electrophoresed for 90 Minutes at 80 Volts
Antisera: Gibco, Rabbit Anti Whole Human, Lot #30N9011

Figure 11

Immunoelectrophoresis of IVBG Final Products Precipitated with Anti Human IgA



	mg/ml
1 * IVBG-1A	103
2 * IVBG-1B	108
3 * IVBG-2A	105
4 * IVBG-2B	103
5 ▲ IVBG-2A	105
6 ▲ IVBG-1B	108
7 ▲ IVBG-1A	103

Samples Electrophoresed for 80 Minutes at 90 Volts

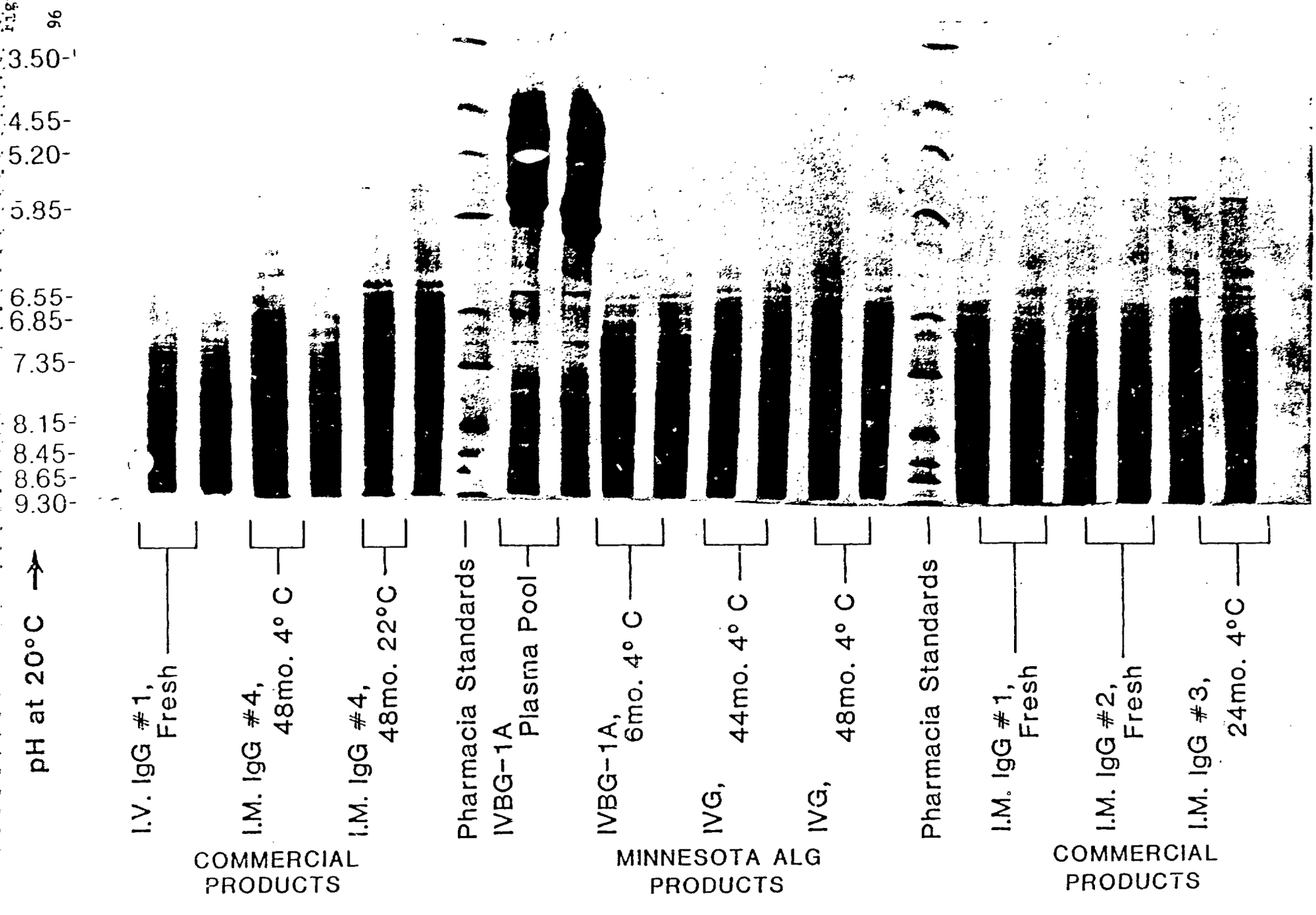
Antisera: Kallestad, Goat Anti Human IgA

* Diluted 1:64 with NaCl

▲ Diluted 1:128 with NaCl

Figure 12

Analytical Isoelectric Focusing of IVBG-1A and Other Human IgG Preparations in a Polyacrylamide Thin Layer Gel pH 3.5 – pH 9.5



Analytical Isoelectric Focusing of IVBG-1B in a Polyacrylamide Thin Layer Gel pH 3.5 – pH 9.5

Figure 13

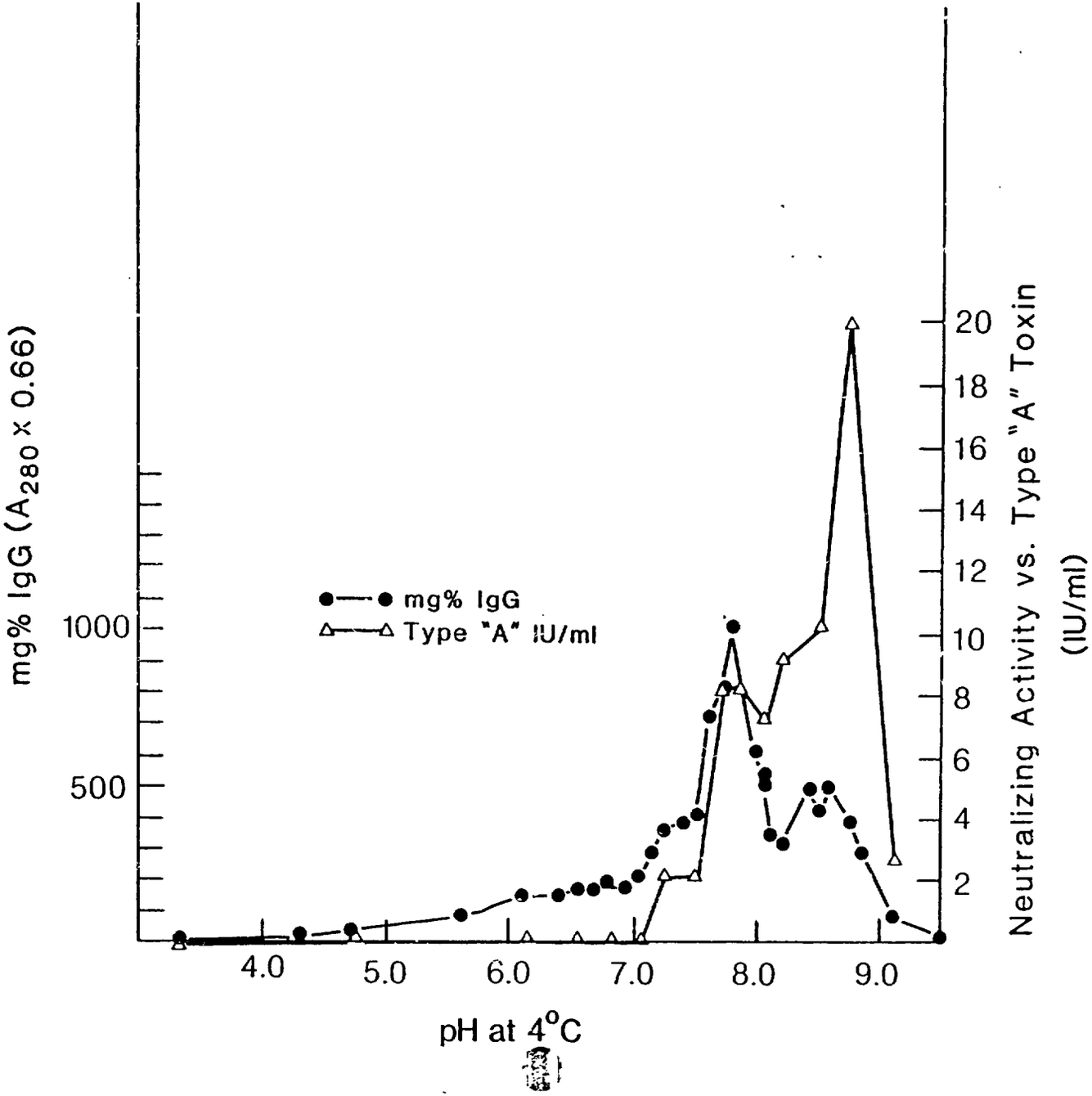
Analytical Isoelectric Focusing of IVBG-2A in a Polyacrylamide Thin Layer Gel pH 3.5 – pH 9.5

Figure 14

Analytical Isoelectric Focusing of IVBG-2B
in a Polyacrylamide Thin Layer Gel pH 3.5 – pH 9.5

Figure 16

Preparative Isoelectric Focusing of IVBG-1A Final Product: IgG Concentration and Neutralizing Activity vs. Type "A" Toxin



High Pressure Liquid Exclusion Chromatography
of IVBG Final Products

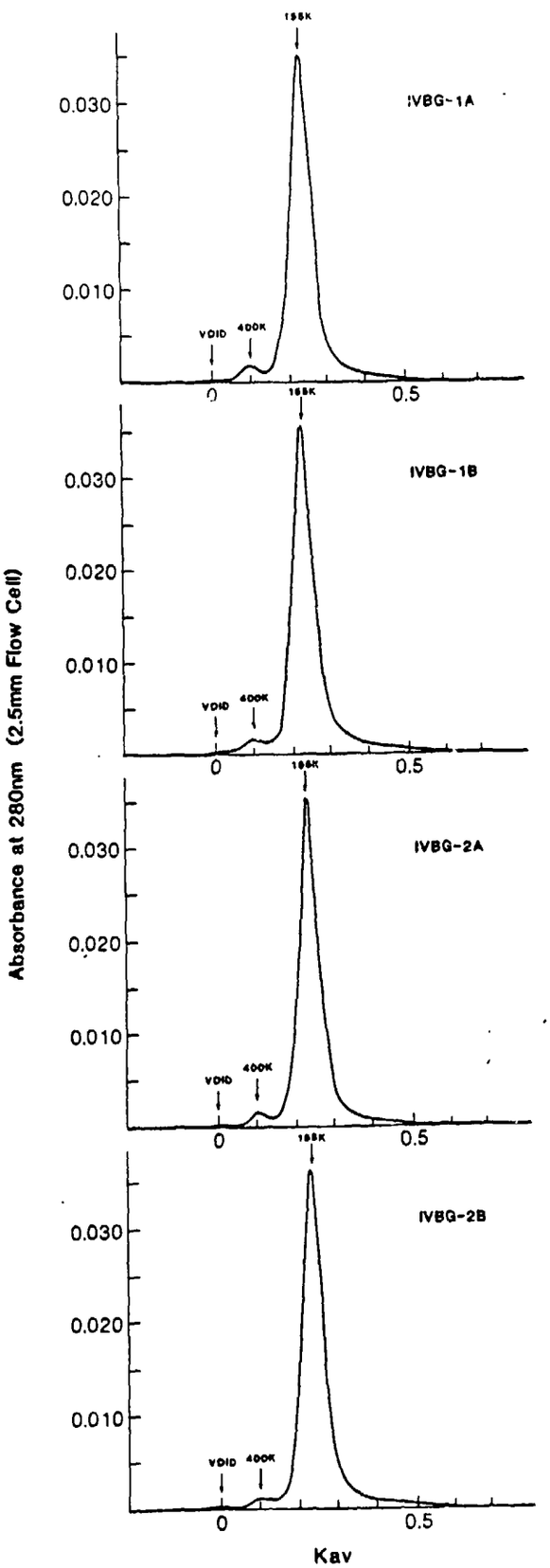


Figure 18

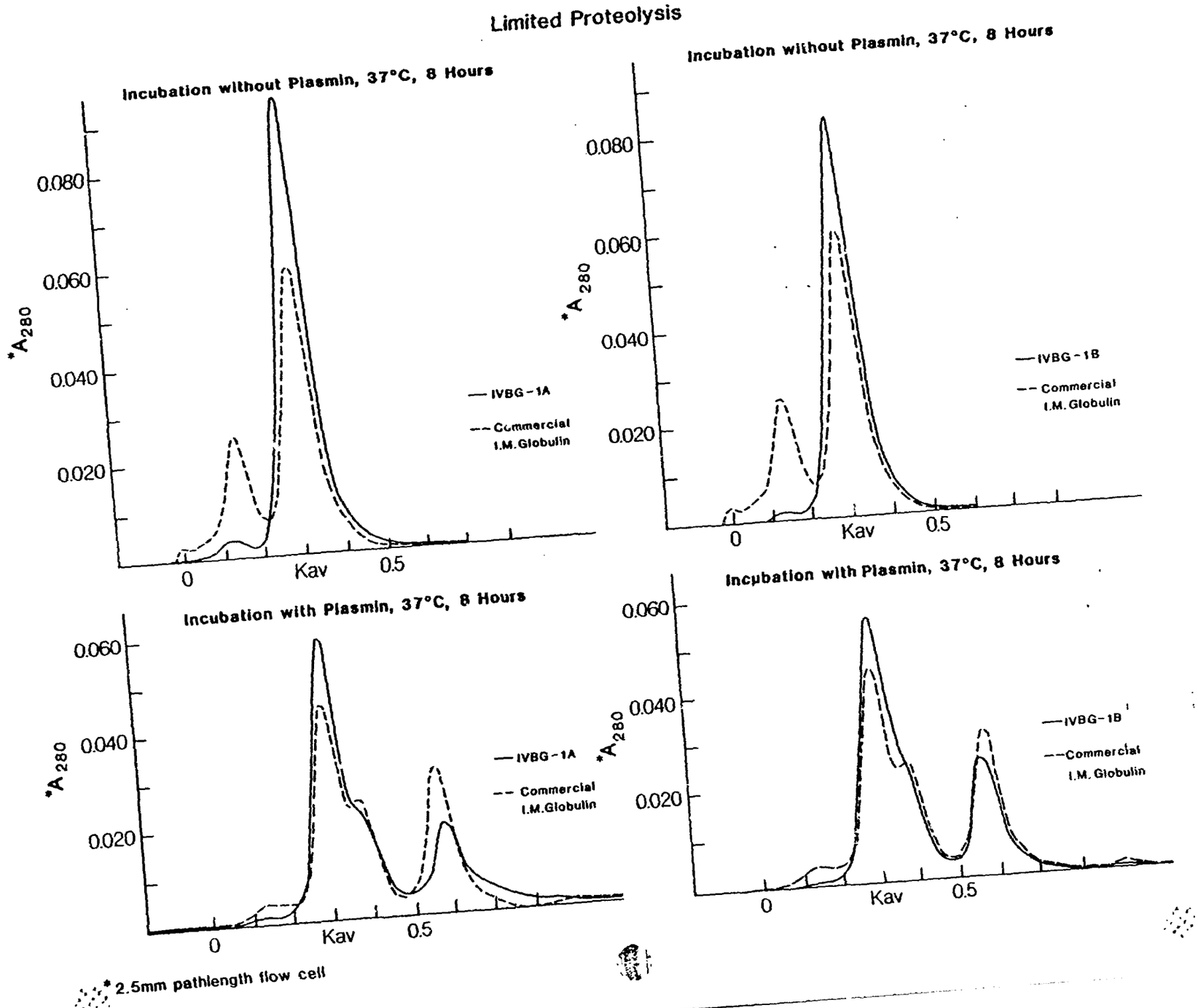
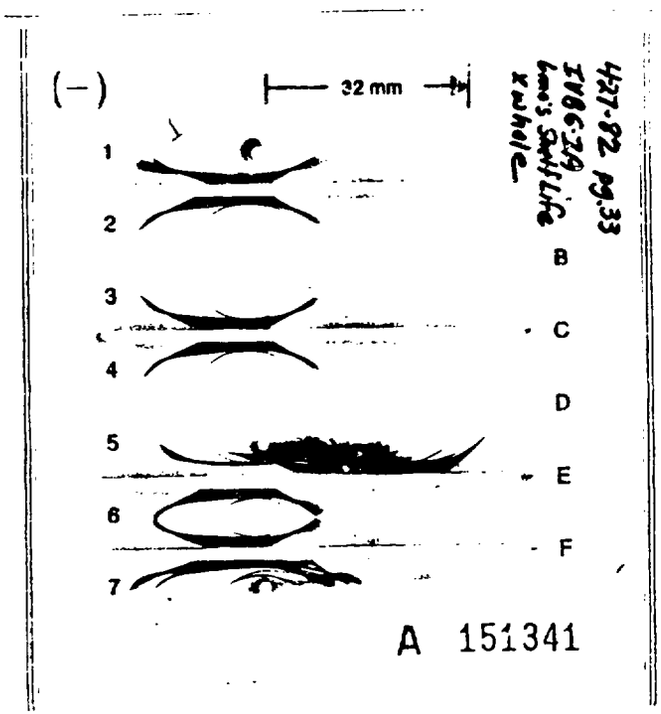

Limited Proteolysis
Incubation without Plasmin, 37°C, 8 Hours
*A280
0.080
0.060
0.040
0.020
0
Kav
0.5
IVBG-1A
Commercial I.M.Globulin
Incubation without Plasmin, 37°C, 8 Hours
IVBG-1B
Commercial I.M.Globulin
Incubation with Plasmin, 37°C, 8 Hours
IVBG-1A
Commercial I.M.Globulin
Incubation with Plasmin, 37°C, 8 Hours
IVBG-1B
Commercial I.M.Globulin
* 2.5mm pathlength flow cell

Figure 19
103

SHELF LIFE STUDY
Immunoelectrophoresis of IVBG-1A Six Month Samples



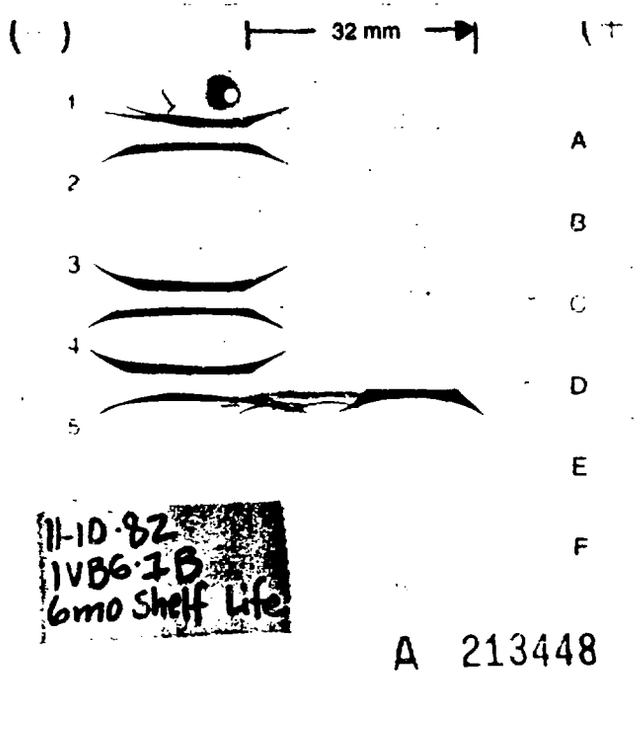
			mg/ml
1	IVBG-1A	37°C	103.0
2		RT	103.0
3		4°C	103.0
4		-20°C	103.0
5	Normal Human Serum		As Is
6	209-IVG, Minnesota IgG		55.5
7	Gammar®, Armour IgG		100.0

Lot # T10302

Samples Electrophoresed for 90 Minutes at 80 volts
Antisera: Kallestad, Rabbit Anti Whole Human, Lot #301L011

Figure 20

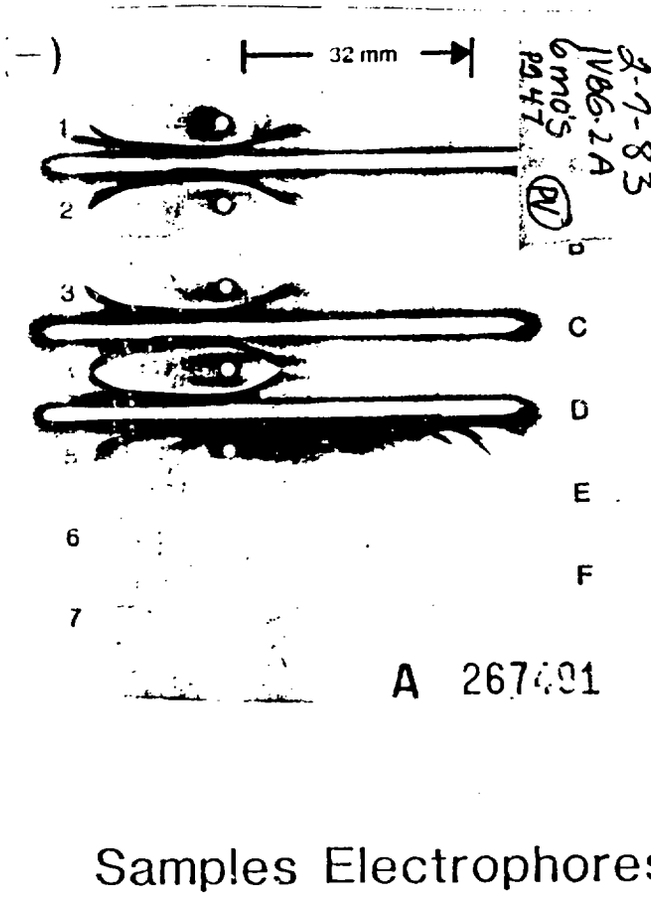
SHELF LIFE STUDY
Immunoelectrophoresis of IVBG-1B Six Month Samples



			mg/ml
1	IVBG-1B	37°C	108
2		RT	108
3		4°C	108
4		-20°C	108
5	Normal Human Serum		As Is
6			
7			

Samples Electrophoresed for 90 Minutes at 80 Volts
Antisera : Gibco Rabbit Anti Whole Human, Lot #27P8023

SHELF LIFE STUDY
Immunoelectrophoresis of IVBG-2A Six Month Samples

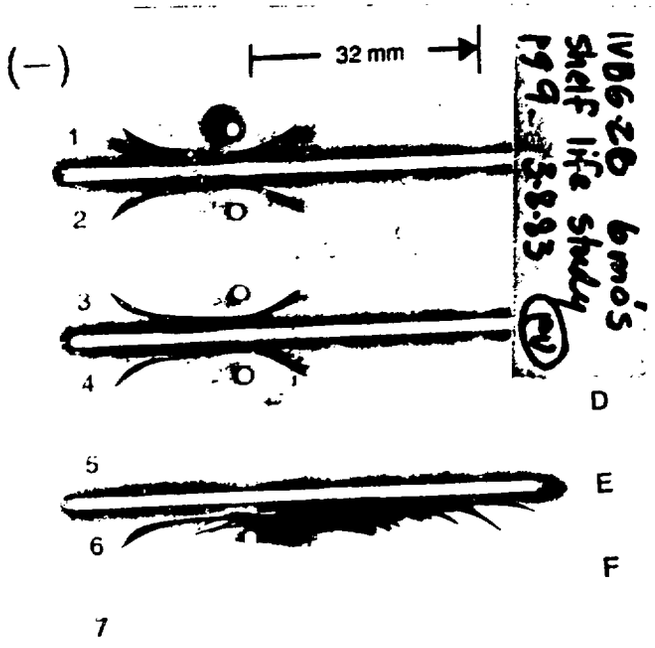


			mg/ml
1	IVBG-2A	37°C	105
2		RT	105
3		4°C	105
4		-20°C	105
5	Normal Human Serum		as is
6			
7			

Samples Electrophoresed for 90 Minutes at 80 Volts
Antisera: Kallestad, Goat Anti Whole Human, Lot #201N014

Figure 22

SHELF LIFE STUDY
Immunoelectrophoresis of IVBG-2B Six Month Samples



A 275764

			mg/ml
1	IVBG-2B	37°C	103.0
2		RT	103.0
3		4°C	103.0
4		−20°C	103.0
5			
6	Normal Human Serum		as is
7			

Samplers Electrophoresed for 90 Minutes at 80 Volts
Antisera: Kallestad, Goat Anti Whole Human, Lot #201N014

Figure 23

SHELF LIFE STUDY

Analytical Isoelectric Focusing of IVBG-1A Six Month Samples

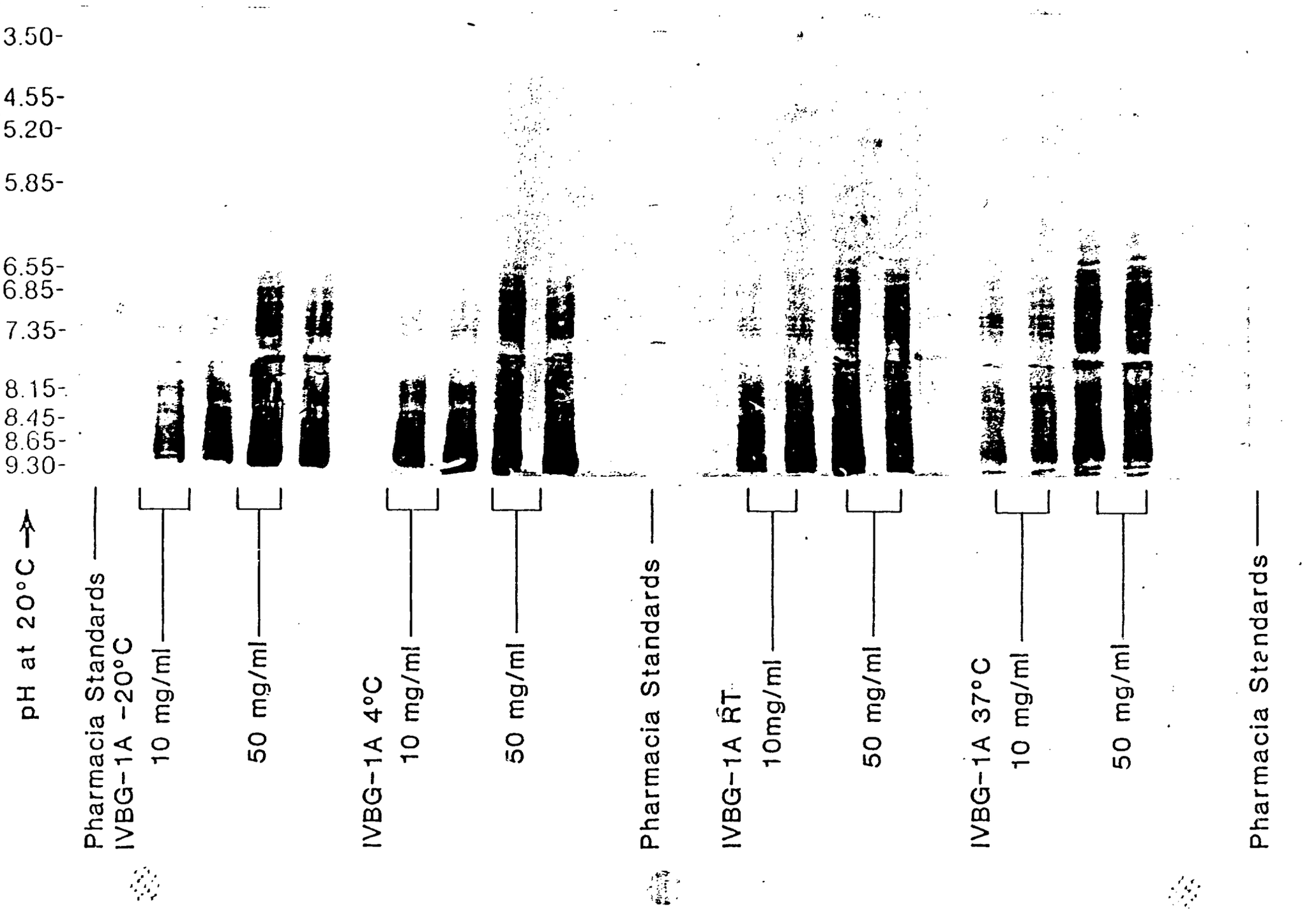


Figure 24

Analytical Isoelectric Focusing of IVBG-1B Six Month Samples

SHELF LIFE STUDY

Figure 25

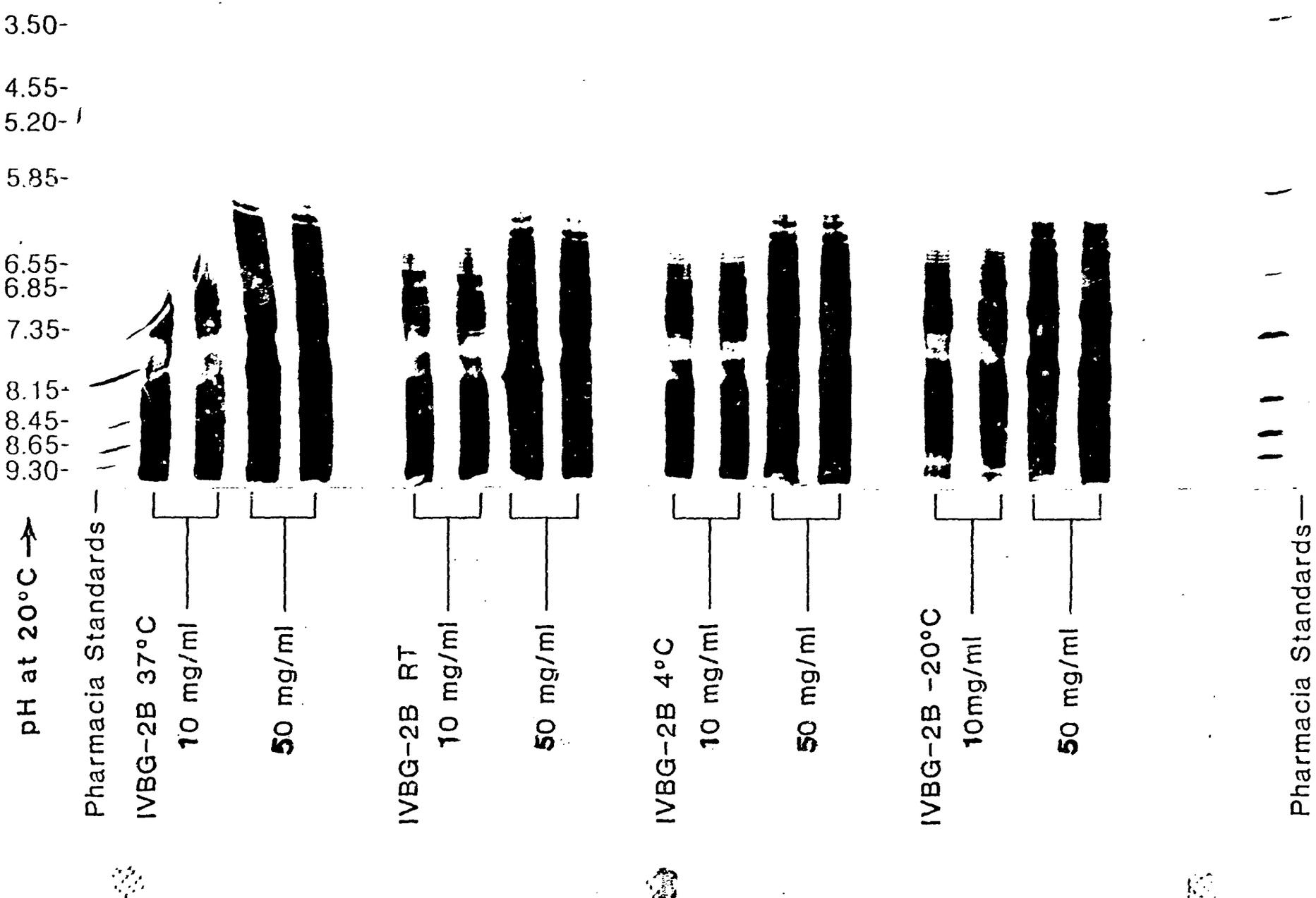
Analytical Isoelectric Focusing of IVBG-2A Six Month Samples

SHELF LIFE STUDY

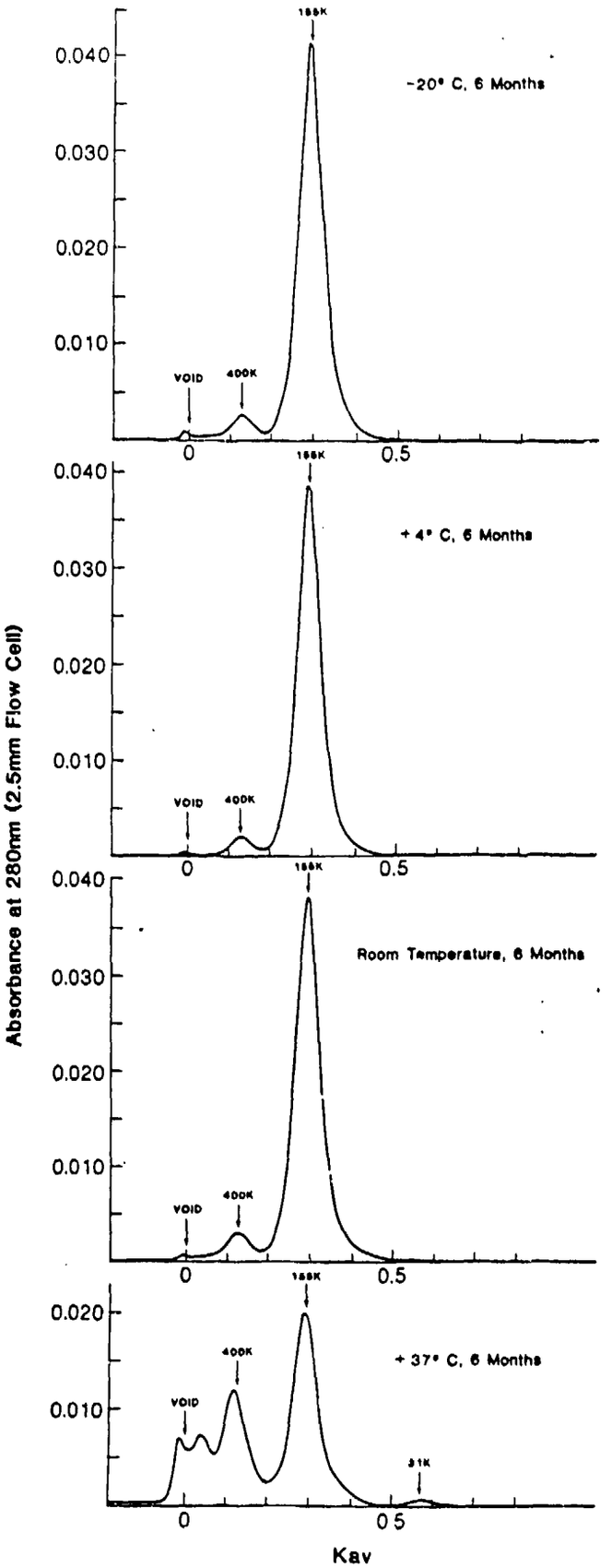
Figure 26

SHELF LIFE STUDY

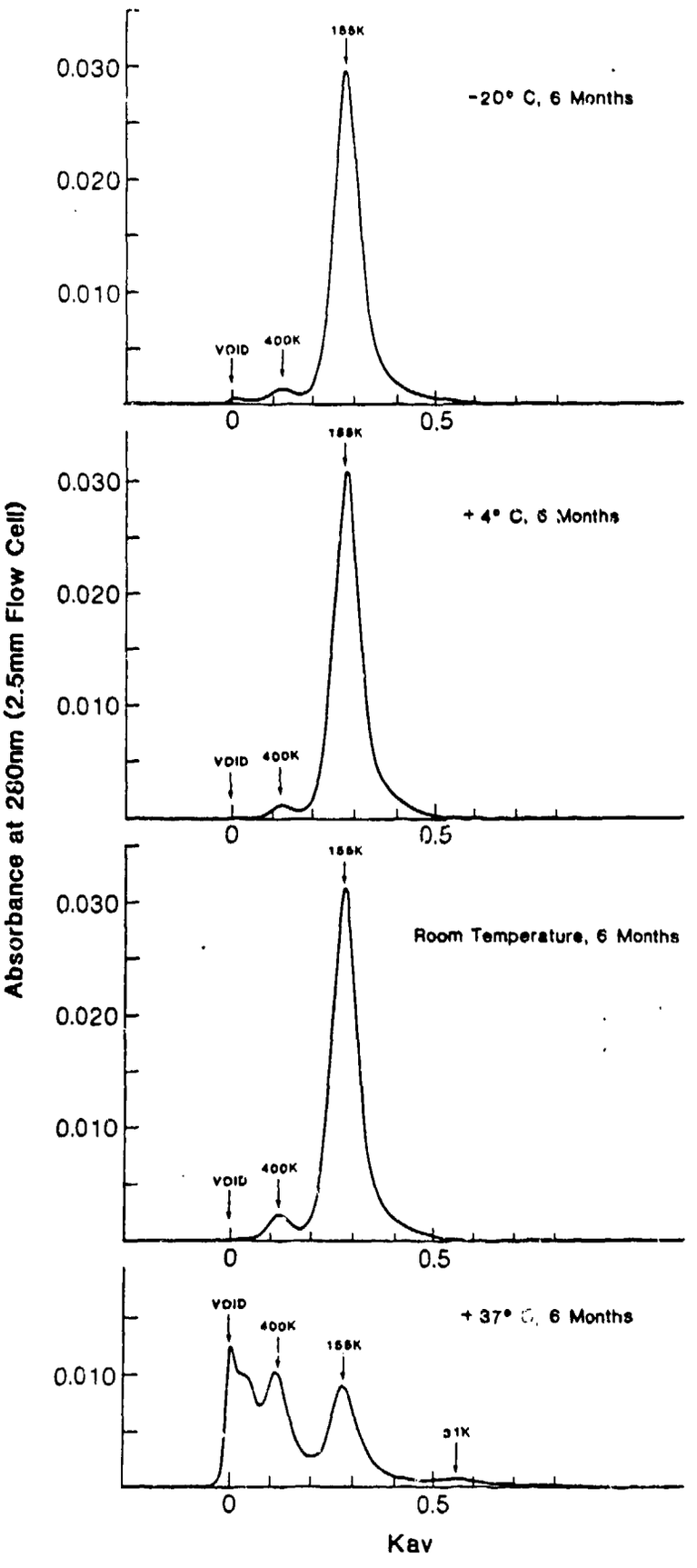
Analytical Isoelectric Focusing of IVBG-2B Six Month Samples



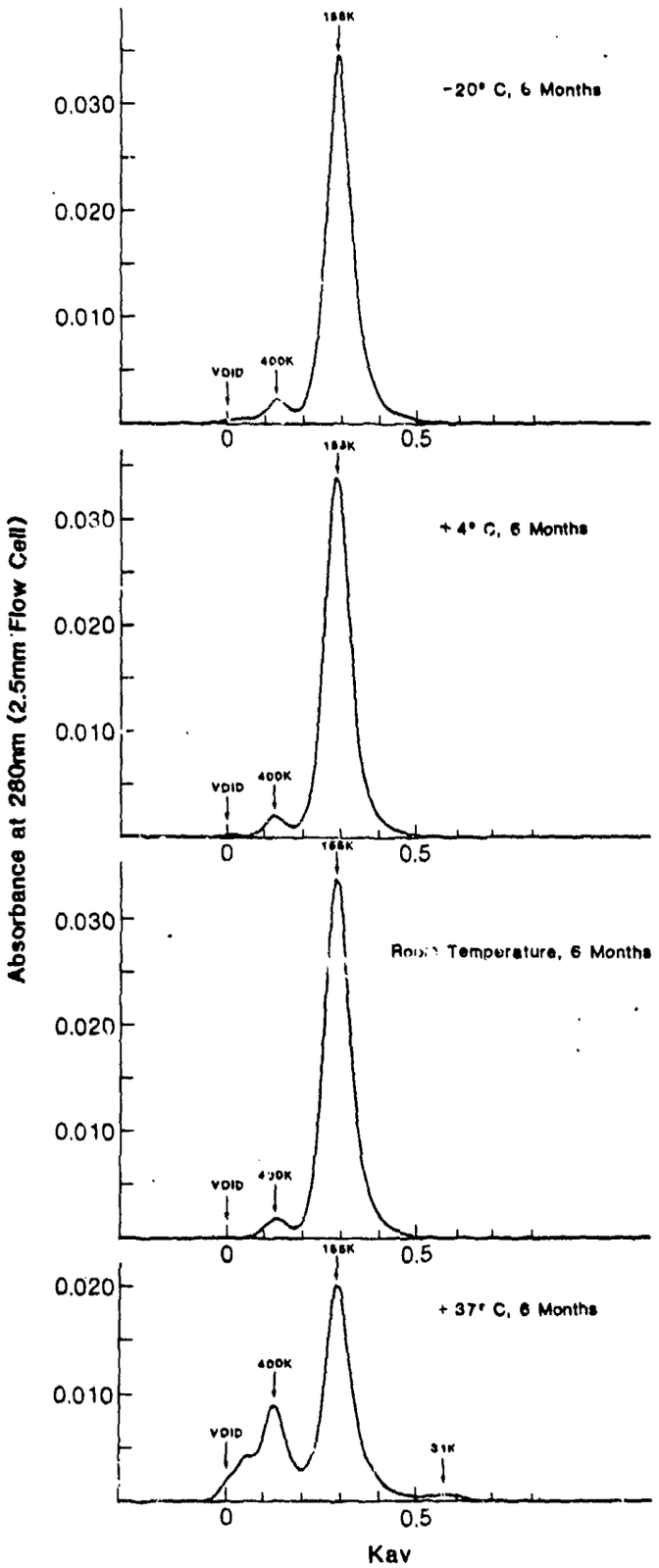
SHELF LIFE STUDY
High Pressure Liquid Exclusion Chromatography
of IVBG-1A Stored at Various Temperatures



SHELF LIFE STUDY
High Pressure Liquid Exclusion Chromatography
of IVBG-1B Stored at Various Temperatures

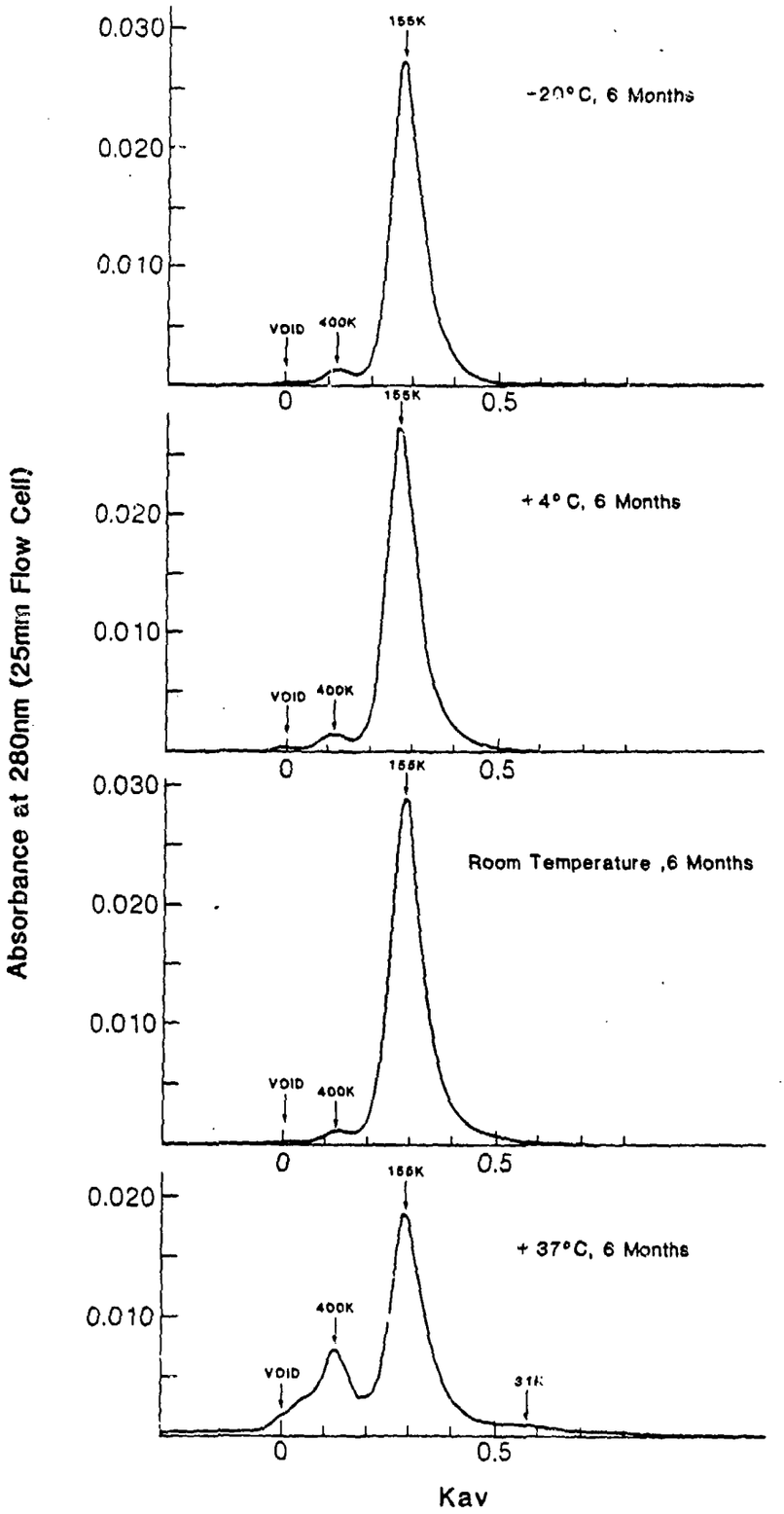


SHELF LIFE STUDY
High Pressure Liquid Exclusion Chromatography
of IVBG-2A Stored at Various Temperatures



SHELF LIFE STUDY

High Pressure Liquid Exclusion Chromatography
of IVBG-2B Stored at Various Temperatures



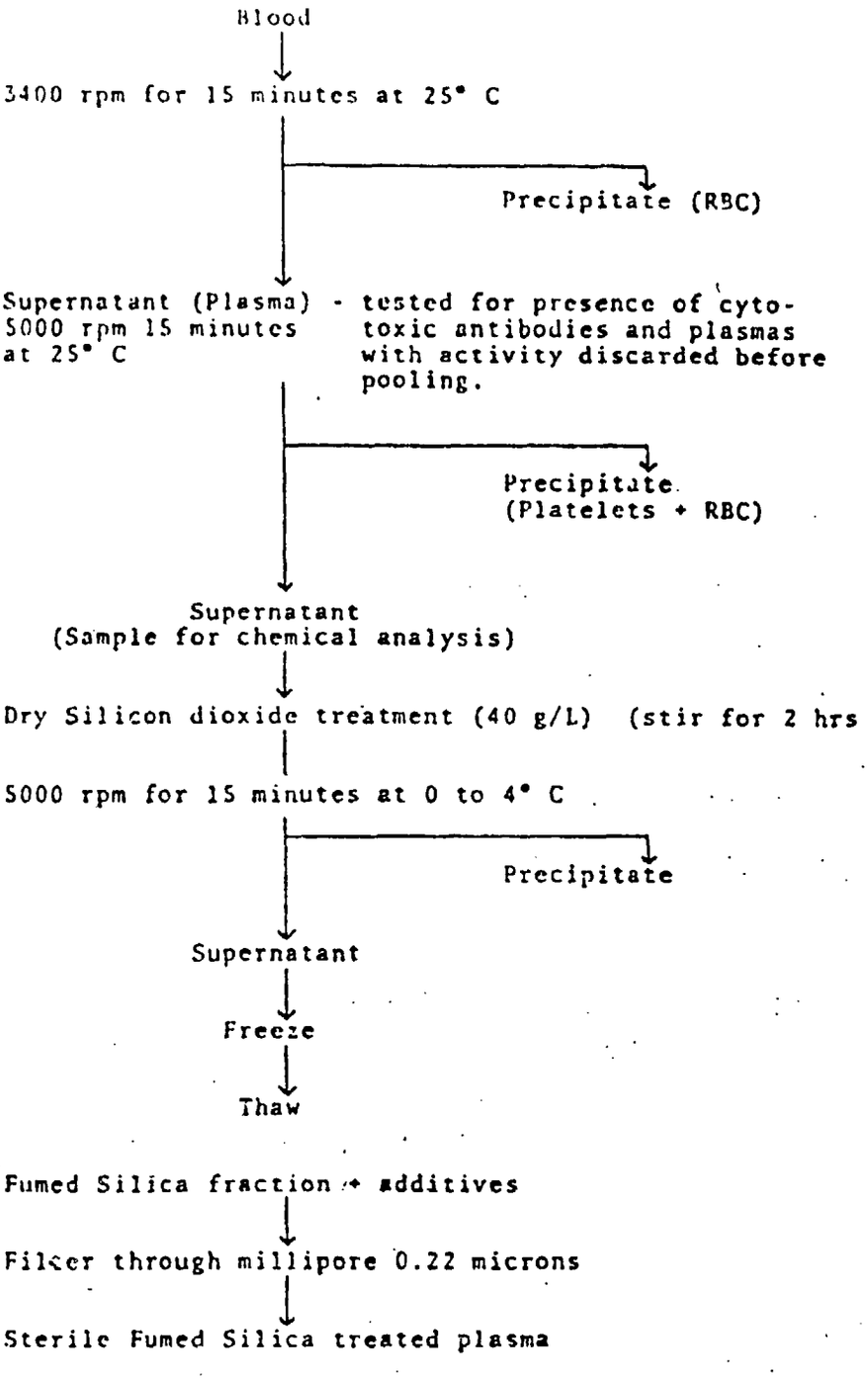

Blood
3400 rpm for 15 minutes at 25° C
Precipitate (RBC)
Supernatant (Plasma) - tested for presence of cyto-toxic antibodies and plasmas with activity discarded before pooling.
5000 rpm 15 minutes at 25° C
Precipitate (Platelets + RBC)
Supernatant (Sample for chemical analysis)
Dry Silicon dioxide treatment (40 g/L) (stir for 2 hrs
5000 rpm for 15 minutes at 0 to 4° C
Precipitate
Supernatant
Freeze
Thaw
Fumed Silica fraction + additives
Filter through millipore 0.22 microns
Sterile Fumed Silica treated plasma

FIG. 1

Appendix A1

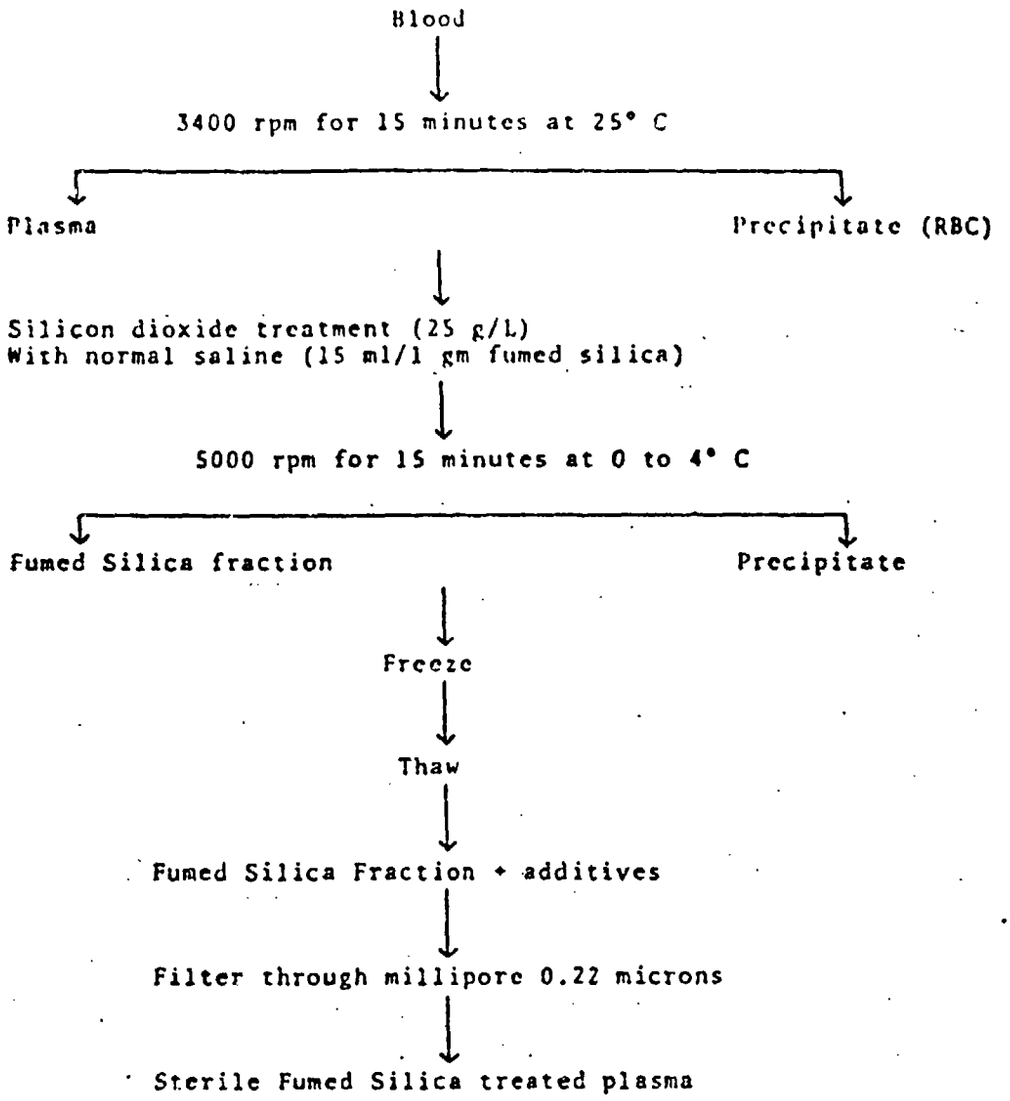


FIG. 2

[54] **FIBRINOGEN-FREE PLASMINOGEN-PLASMIN-FREE PLASMA AND METHOD OF PREPARING AND USING SAME**

[75] Inventors: Richard M. Condie, Minneapolis, Luis H. Toledo-Pereyra, Hopkins, both of Minn.

[73] Assignee: The Regents of the University of Minnesota, Minneapolis, Minn.

[22] Filed: Apr. 23, 1975

[21] Appl. No.: 570,569

[52] U.S. Cl. 424/101
[51] Int. Cl.[2] A61K 35/16; A61K 35/14
[58] Field of Search 424/101

[56] References Cited

OTHER PUBLICATIONS

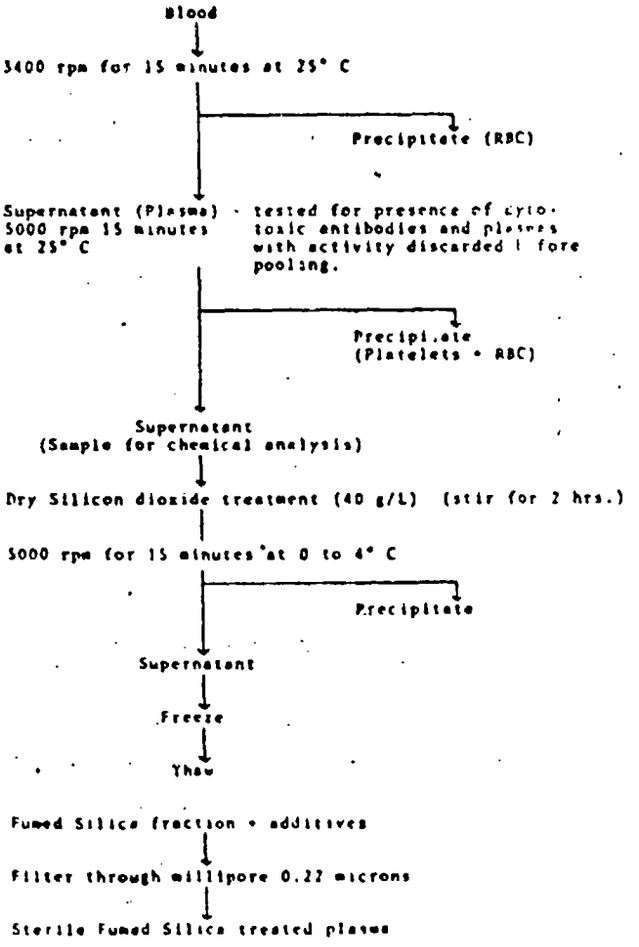
Stephan et al., Chem. Abst., vol. 69 (1968), p. 17412h.

Primary Examiner—Sam Rosen
Attorney, Agent, or Firm—Burd, Braddock & Bartz

[57] ABSTRACT

A method of treating blood plasma or fractionated plasma products with fumed colloidal silica to remove fibrinogen without polymerization to fibrin, to remove the plasminogen-plasmin proteolytic enzyme system, to remove cholesterol and lipoproteins and reduce triglycerides, while maintaining plasma coagulation factor II at pretreatment levels and leaving immunoglobulins and other protein constitutents unaffected, and the resulting product. Plasma products treated with fumed silica may be subjected to long-term storage for a year or more without loss of its biologically active support properties, thereby circumventing the problem of hepatitis. The treated plasma products, either fresh or after long-term storage, may be used as a perfusion support media for organ perfusion, for treatment of hemmorrhagic shock and similar purposes for which untreated plasma and fractionated plasma products are customarily used, with equal or superior effectiveness.

2 Claims, 2 Drawing Figures



FIBRINOGEN-FREE PLASMINOGEN-PLASMIN-FREE PLASMA AND METHOD OF PREPARING AND USING SAME

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education and Welfare.

This invention is directed to a fibrinogen-free plasminogen-plasmin-free blood plasma product and to the method of making such a product by the treatment of animal blood plasma or fractionated plasma products with a pure synthetic fumed colloidal silicon dioxide containing siloxan and silanol groups on its surface. It has been found that fumed silica treated plasma product retains its biological support properties through longterm storage and is useful in organ perfusion, treatment of shock, and the like, while circumventing the transmission of hepatitis in untreated plasma.

BACKGROUND OF THE INVENTION

With the advent of clinical organ transplantation as an accepted method of treatment for various diseases, the need for preservation of cadaver organs became established. The requirements of a media that will support organ perfusion were partially accomplished by the use of cryoprecipitated plasma (CPP). Thus, the problems associated with blockage of capillaries by lipoproteins, fibrin aggregates, and the like, were to a certain degree prevented. There are, however, several disadvantages related to the use of CPP. (a) it is difficult to standardize and store; (b) it must be thawed and filtered immediately before use; and (c) there is a risk of transmission of hepatitis in the use of unstored plasma. Most important, it is not reliable for long-term perfusion since renal function does not reliably and immediately reappear upon transplantation of kidneys preserved for more than 48 hours.

A number of efforts have been made to eliminate some of these disadvantages. In particular, the use of synthetic plasma derivatives and albumin solutions have been tried. Such solutions theoretically should contain little aggregated materials, i.e., lipoproteins and partially polymerized fibrinogen and since they can be stored there is little risk of hepatitis. However, for unknown reasons these materials are not as satisfactory as plasma. In applicants' laboratory at the University of Minnesota Medical School, canine kidneys perfused with albumin demonstrated only 50 percent survival after 48 hours preservation. Furthermore, only 25 percent of the kidneys perfused with plasmanate survived after the same preservation period.

One possible explanation why CPP is superior to the other plasma derivatives for organ perfusion is the trace materials within plasma which are necessary for metabolic maintenance of the hypothermic kidney. Such materials may be removed by the processing of albumin or plasmanate. At the same time it may be possible that the problem with CPP is related to the failure to remove by freezing all of the precipitable and denaturable elements within the plasma.

The picture for shock is even less clear. Upon storage, plasma and blood lose their effectiveness as plasma expanders in hypovolemic and endotoxic shock. Serum, the protein fluid remaining after the polymerization of fibrinogen, is of little value in organ perfusion and may be even toxic, whereas, in shock, even saline is more effective than serum, suggesting that in the clotting of blood harmful pharmacological active factors may be generated.

The deficiencies in the above mentioned preparations led to the investigation and development of the method of the present invention for (1) removing fibrinogen from plasma without its polymerization to fibrin and leaving prothrombin at pretreatment levels, (2) removing the plasminogen-plasmin proteolytic enzyme system, (3) without generation of toxic factors nor removal of biologically essential support factors, and (4) allowing for long-term storage of plasma without loss of its biological support properties.

A series of investigations with various natural and synthetic poly-silica compounds, resulted in the discovery that a synthetic fumed colloidal silicon dioxide could be used to treat plasma removing fibrinogen without polymerization, and preventing the accumulation of pharmacologically active toxic split products. Subsequent research led to the discovery that in addition the proteolytic enzyme system plasminogen-plasmin is also removed by fumed silica treatment. Plasma treated and processed in this manner has been extensively tested in both animal and human models and evaluated for efficacy for organ preservation, shock, and for retention of its biological support properties following long-term storage (1 year).

Blood contains plasminogen, the inactive precursor of the potent proteolytic enzyme plasmin. Under ordinary conditions such as those involved in the preparation of plasma from citrated whole blood, plasminogen is not activated in any detectable quantities since there are inhibitors in plasma which can act to block activation by kinases as well as inhibitors that can block the action of the enzyme plasmin. However, on long-term storage of plasma there apparently is some activation of plasminogen to plasmin (up to 5 percent) and the subsequent proteolysis of fibrinogen, immunoglobulins, and other proteins. With plasma develops visible aggregates which are presumably products of partially degraded fibrinogen and immunoglobulins. This problem is largely circumvented by the outdating of plasma, resulting in discarding and loss of a valuable resource.

In the classic Cohn alcohol fractionation of human plasma, the plasminogen is concentrated and freed of its inhibitors in the fraction process. The result is that fractions I, II, III of the Cohn procedure contain much greater quantities of plasminogen than are present initially in plasma. The presence of high concentrations of plasminogen, if activated, leads directly to the degradation of fibrinogen to form the toxic so-called split products. In addition, it has been demonstrated that the plasmin system can partially degrade the immunoglobulins, a process which leads to formation of molecular aggregates.

Immunoglobulins acted upon by plasmin when injected into the circulation are eliminated very rapidly when compared to normal, unaltered immunoglobulins. The net effect is to prevent the attainment of high blood levels that are necessary in treating bacterial, toxic states and viral diseases. In particular, the plasmin altered immunoglobulin when administered to immune deficiency patients intravenously produce anaphylactoid-like reactions, thereby eliminating a potentially effective method of treating such patients.

A major obstacle to the preparation of potent solutions of purified immunoglobulins that can be safely administered intravenously to patients to achieve high blood levels in treating immune defects and life threat-

ening infections has been the failure to either eliminate or prevent the aggregation of immunoglobulins during purification. Aside from the fact that the universally employed Cohn alcohol method of plasma protein fractionation may irreversibly denature some plasma proteins, the presence of the plasma proteolytic enzyme plasminogen has been demonstrated to attack, and particularly degrade by its proteolytic activity, immunoglobulins, particularly the IgG class. The IgG immunoglobulins that have been attacked by plasmin form molecular aggregates which have been implicated in the activation of the kinen and complement systems and further, when their aggregated solutions are administered intravenously to patients with immune deficiency, precipitate anaphylactoid systemic reactions In addition, these partially degraded aggregated IgG preparations are rapidly eliminated from the circulation thereby significantly reducing the effectiveness of specific antibody in conferring protection to toxic states resulting, for example, form diphtheria toxin, and protection against infection. Thus, the hoped for goal of achieving high effective blood levels of a biologically active antibody to toxins, viral or bacterial organisms have not been attained to date.

DESCRIPTION OF THE PRIOR ART

In the past there has been no single process that would remove both lipoproteins and fibrinogen without polymerizing to fibrin Cryoprecipitation of plasma has been extensively utilized in the removal of some lipoprotein aggregates and some polymerized fibrinogen Serum separated from clotted whole blood has very low levels of fibrinogen but it is actually toxic in organ perfusion, and harmful in hypovolemic and endotoxic shock.

Intravenous preparations of purified immunoglobulins have been prepared but have all produced reactions when administered intravenously to the immune deficient patient. One preparation prepared by enzymatic treatment with trypsin of IgG, while producing fewer reactions when administered intravenously, had greatly reduced half life. It is presumed that the major obstacles to preparation of a highly purified intravenous IgG has been the presence of plasminogen-plasmin contaminating the early fractionation stages during purification of the immunoglobulins. The major approach to dealing with the plasminogen problem has been the addition of compounds which do not remove the enzyme precursor or its activated plasmin but inhibit the activation of the plasminogen to plasmin. This has come about by application of the fact that E amino caproic acid combines and inhibits the activation of plasminogen to plasmin. It is not clear, however, that this process offers much in the large scale production and preparation of plasminogen free plasma that could be used in preparing aggregate-free plasma protein of high purity, particularly since it has not been demonstrated conclusively that plasmin is inhibited by E amino caproic acid. Purification by chromatography could remove plasminogen. However, it is much more desirable that a process be developed where whole plasma could be treated economically and with ease and in a manner that plasminogen and plasmin could be removed in the early phases of preparation when the active and potent plasma inhibitors of the plasminogen activation system are present and effective in the whole plasma.

Synthetic fumed silicon dioxide has been used to remove lipoproteins, cholesterol and triglycerides from serum However, we have demonstrated that treating plasma with synthetic fumed silicon dioxide removes lipoproteins, cholesterol and triglycerides and, at the same time, removes fibrinogen by mechanisms not involving polymerization of fibrinogen to fibrin since the prothrombin remains at pretreatment levels, and removes the plasminogen-plasmin system

SUMMARY OF THE INVENTION

Broadly stated, the invention comprises the method of treating blood plasma or plasma fractions, human or non human, in the course of normal processing with pyrogenic or fumed silica (colloidal synthetic silicon dioxide of highest purity containing siloxan and silanol groups on its surface), in either wet or dry form, followed by separation of the silica, the resulting fibrinogen free plasminogen-plasmin-free plasma products, and methods of using those products.

The results of this method of plasma treatment provides plasma that can be stored for two years or more without loss in its biological support activities for treatment of hemorrhagic and endotoxic shock and as a support media for organ perfusion. The advantages of long-term storage provides an opportunity for extensive testing, circumventing of hepatitis problems and successful pooling of a valuable natural resource.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is illustrated in the accompanying drawings in which:

FIG 1 is a flow sheet showing the method of preparing fumed silica treated plasma using dry silica; and

FIG 2 is a flow sheet showing a modified form of treatment utilizing saline wetted fumed silica.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Referring now to the drawings, and particularly to FIG. 1, there is illustrated in flow sheet form a preferred method for preparing fumed silicon dioxide treated plasma using dry fumed silica. The drawn blood is first treated in the coventional manner by centrifuging at about 3400 rpm for about 15 minutes at about 25° C. The precipitate consisting predominantly of red blood cells is separated. The plasma, the supernatant fraction, is tested for the presence of cytotoxic antibodies. Any plasma with such activity is discarded. The remaining blood is pooled for further processing. The plasma is centrifuged at about 5000 rpm for about 15 minutes at about 25° C. The precipitate composed predominantly of platelets and red blood cells is again separated. A sample of the supernatant plasma is withdrawn for chemical anaylsis.

Sterile dry colloidal fumed silicon dioxide of the highest purity is then added to the plasma at room temperature with stirring to a final concentration of between about 10 to 50 and preferably about 25 to 40 gram of silica per liter of plasma Exemplary pyrogenic or fumed silicas which may be used are those sold under the brand name Aerosil 380 by Degussa, Inc., New York, N Y , and under the brand name Cab-O-Sil by Cabot Corp , Boston, Mass The plasma and silica are stirred for from about 20 minutes to 2 hours from 4° C to 37° C but preferably at room temperature, and then centrifuged at about 100 to 10000 rpm for about 10 to 30 minutes, preferably at about 5000 rpm for

about 15 minutes, at a temperature in the range between about 0° to 37° C, preferably about 0° to 4° C. The precipitate comprising the silica and removed constituents is again separated and the supernatant plasma may optionally be frozen for storage. Any desired additives may be admixed. The product is filtered through a 0.22 micron bacterial filter and is ready for use or storage under sterile conditions without preservatives either at room temperature or at 4° C. Although centrifugal separation is preferred, the supernatant liquid may be separated by filtration.

According to the modified form of treatment illustrated in FIG. 2, the drawn blood is centrifuged at about 3400 rpm for about 15 minutes at about 25° C and the precipitate separated. After testing and pooling, sterile fumed silica in admixture with normal saline in concentration of about 1 gram silica per 15 milliliters of saline solution is added to the plasma with stirring at room temperature to a final concentration between about 10 to 50 and preferably about 25 to 50 grams silica per liter of plasma. The mixture is stirred from 20 minutes to 2 hours and thereafter centrifuged or filtered, as described. The silica treated supernatant fraction, after separation of the precipitate, is handled as previously described.

Horse, dog, rabbit and human plasma have been treated with fumed silica according to the described methods. The treatment of plasma in this manner completely removes fibrinogen without liberation of split products or other pharmacologically active poly-peptide products. It removes the plasminogen-plasmin proteolytic enzyme system. It removes all of most of the aggregateable beta-lipoproteins and thermo-denaturable lipoproteins, fats and lipids, i.e., cholesterol, triglycerides, etc. In addition, the fumed silicon dioxide has been shown to remove bacteria and herpes virus, traces of red blood cell membranes, white blood cells and platelets. The addition of fumed silica to plasma does not have any effect on concentrations and activities of the enzymes LDH, SGOT, and beta-glucuronidase, nor does it modify the level or activities of the immunoglobulins IgG, IgM, IgA. Plasma coagulation factor II is left at pretreatment level.

The removal of those most labile materials which on storage at 4° or 25° C either are denatured or form aggregates permit long-term storage of the silica treated plasma without loss of the biologically active support properties of the plasma. A comparative chemical analysis of untreated dog plasma with cryoprecipitation treated plasma and silica treated plasma is shown in Table I.

TABLE I

COMPARATIVE CHEMICAL ANALYSIS OF THE DOG PLASMA TREATED WITH CRYOPRECIPITATION ALONE AND THE DOG PLASMA EXTRACTED WITH FUMED SILICA (Mean values ± SE)

Chemical Constitution	Untreated Plasma	Cryoprecipitated Plasma	Silica Fraction
Cholesterol (mg%)	100.7 ± 15.3	88.7 ± 9.6	0
Triglycerides (mg%)	59.9 ± 11.5	62.8 ± 8.5	20.3 ± 6.5
Fibrinogen (mg%)	0.41 ± 0.17	0.26 ± 0.11	0
α-Lipoproteins	Normal trace	Normal trace	0
β-Lipoproteins	Normal trace	Normal trace	0
Free Fatty Acids (mEq/L)	0.6 ± 0.2	0.4 ± 0.2	0.35 ± 0.15
Total Proteins (gm%)	3.2 ± 0.2	2.9 ± 0.3	3.1 ± 0.4
Albumin (gm%)	2.5 ± 0.2	1.8 ± 0.2	2.0 ± 0.3
Globulins (gm%)	0.6 ± 0.4	1.1 ± 0.2	1.1 ± 0.1
Osmolarity (mOsm/L)	292 ± 7.3	285 ± 5.1	290 ± 6.7
Sodium (mEq/L)	139 ± 4.3	137 ± 5.2	140 ± 3.5
Potassium (mEq/L)	4.2 ± 0.4	4.3 ± 0.6	4.1 ± 0.6
Chloride (mEq/L)	99 ± 6.7	98 ± 6.5	100 ± 5.7

Extensive testing has been conducted in both animal and human models to evaluate the efficacy of fumed silica treated plasma for use in organ preservation, treatment of shock, and for retention of biological support properties following long term storage. These studies demonstrate that silica treated plasma is superior to CPP in the preservation of dog kidneys for 48 to 120 hours. Furthermore, silica treated plasma stored for one year at room temperature still served as a superior perfusate during 48 hour preservation. In dog kidneys perfused by hypothermic pulsatile perfusion for 48 hours to 120 hours comparing the silicon dioxide treated plasma with standard cryoprecipitated plasma (CPP), it was revealed that the chemical characteristics of the perfusate and the physical characteristics of perfusion were more stable when silicon dioxide treated plasma was used. Survival was consistently better following autotransplantation of the preserved kidney and contralateral nephrectomy when the perfusate was silicon dioxide treated plasma. Two of eight kidneys perfused for 120 hours with silicon dioxide treated plasma had returned to normal renal function by 18 days after autotransplantation. Perfusion was according to the system of Moberg et al described in Lancet, 1971, 2:1403. These results are reported in *Surgery, Gynecology & Obstetrics*, June 1974, Vol. 138, pp. 910–905, incorporated herein by reference.

The finding that fumed silica treatment of plasma removes the plasminogen-plasmin system in addition to fibrinogen, cholesterol, fatty acids, lipoproteins, etc. offers additional evidence explaining the superior nature of this processed plasma in organ perfusion. The presence of activatable plasminogen in plasma could among other things lead to the formation of aggregated immunoglobulins, fibrin or fibrinogen split products and the release of pharmacologically active peptides. A more critical factor is the possibility that plasminogen activated by urokinases released from the kidney during perfusion, would activate all the plasminogen resulting in the injury of the endothelial lining of the capillary vessel, which in turn could lead to areas of clot formation at the time the perfused kidney is transplanted and circulation reconstituted.

In the light of findings that use of fumed silica treated plasma results in considerable improvement of the support media for long-term perfusion, as compared with cryoprecipitated plasma, attention was directed to the use of silica treated plasma in the treatment of hypovolemic shock in dogs. Mongrel dogs weighing between 17 to 24 kg were anesthetized with sodium methohexital for induction and halothane for mainte-

nance. They were bled 60% of the blood volume in a one hour period until the systolic blood pressure was 40 mmHg. Thereafter, several groups of dogs were studied (8 dogs per group) according to the type of solution utilized. All dogs but the control group received the same volume of plasma or blood extracted during bleeding. This volume was administered immediately following the one hour of continuous bleeding. Group I, no treatment was given; Group II, treated with Ringer's lactate; Group III, treated with dog plasma; Group IV, treated with human plasmanate; Group V, fresh whole blood plus 20% Ringer's lactate; Group VI, treated with dog's serum; and Group VII, treated with plasma prepared by fumed silica fractionation. The dog's survival was followed for at least three days post reinfusion. Daily IV infusion of 1,000 cc of Ringer's lactate was administered to all survivors. Thereafter, normal feeding was instituted. The results are tabulated in Table II. Silica treated plasma proved to be superior to other protein plasma fractions in the treatment of hypovolemic shock.

TABLE II

HEMORRHAGIC SHOCK

	Groups	Survival
I	Control - No Treatment	12.5% (1/8)
II	Ringer's Lactate	50.0% (4/8)
III	Dog Plasma	75.5% (6/8)
IV	Human Plasmanate	62.5% (5/8)
V	Fresh Whole Blood and 20% Ringer's Lactate	87.5% (7/8)
VI	Serum	12.5% (1/8)
VII	Fumed Silica Fraction	75.5% (6/8)

There are several advantages of fumed silica treated plasma in relation to whole blood or plasma in the treatment of hemorrhagic shock; (1) it can be stored at warm or cold temperatures for long periods of time (one year or more) without deterioration of activity; (2) there are no bacteria found after sterilization and filtering; (3) although virus have not been identified, there is definite evidence the elimination of herpes simplex; and (4) there has been no sensitization observed. Therefore, these characteristics favor the use of silica treated plasma in the treatment of hemorrhagic shock.

Up to the present time, fibrinogen has only partially been removed from plasma by clotting, salt or alcohol fractionation. Treatment of plasma with the synthetic silicon dioxide removes fibrinogen without liberation of fibrin split products or consumption of prothrombin, as well as coagulation factor II, indicating that removal of fibrinogen is not by way of polymerization of fibrinogen to fibrin. Comparison of silicon dioxide treated plasma and untreated plasma and serum is shown in Table III.

TABLE III

FIBRINOGEN REMOVAL WITHOUT POLYMERIZATION TO FIBRIN BY TREATING PLASMA WITH A SYNTHETIC SILICON DIOXIDE

Clotting Factor	Plasma (Normal Activity)	Fumed Silica Reacted Plasma (Per cent Recovery)	Serum (Per cent Recovery)
II	78	103	36
V	100	4	24
VII	45	16*	202
X	61	38	127
VIII	71	4	4
IX	128	28	247
XI	77	5	247
XII	100	4	150
ϕ (Split prod.)	492 mg/100 ml	0	0.19
ϕ*	330 mg/100 ml	0	0

*Radioimmunodiffusion

It is apparent that many modifications and variations of this invention as hereinbefore set forth may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only and the invention is limited only by the terms of the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. In the method of perfusing kidneys removed by nephrectomy to preserve the same pending transplantation into a living body, the improvement which consists in using as a perfusate a fumed silica treated fibrinogen-free, plasminogen-plasmin-free and lipoprotein and lipid-free blood plasma product produced by:

A. intimately admixing finely divided sterile fumed silica containing siloxan and silanol groups at its surface with a blood plasma product selected from the class consisting of blood plasma and plasma fractionation products containing fibrinogen, plasminogen-plasmin enzyme system and lipoproteins and lipids, and

B. separating the silica and associated fibrinogen, plasminogen-plasmin, lipoprotein and lipids from the remaining plasma product.

2. In the method of treating hypovolemic (hemorrhagic) and endotoxic shock in living bodies, the improvement which consists in transfusing the body with a fumed silica treated fibrinogen-free, plasminogen-plasmin-free and lipoprotein and lipid-free blood plasma product produced by:

A. intimately admixing finely divided sterile fumed silica containing siloxan and silanol groups at its surface with a blood plasma product selected from the class consisting of blood plasma and plasma fractionation products containing fibrinogen, plasminogen-plasmin enzyme system and lipoproteins and lipids, and

B. separating the silica and associated fibrinogen, plasminogen-plasmin, lipoprotein and lipids from the remaining plasma product.

* * * * *

United States Patent [19]

Condie

[11] 4,136,094

[45] Jan. 23, 1979

[54] PREPARATION OF INTRAVENOUS HUMAN AND ANIMAL GAMMA GLOBULINS AND ISOLATION OF ALBUMIN

[75] Inventor: Richard M. Condie, Minneapolis, Minn.

[73] Assignee: The Regents of the University of Minnesota, Minneapolis, Minn.

[21] Appl. No.: 829,565

[22] Filed: Aug. 31, 1977

[51] Int. Cl.[2] A23J 1/06; A61K 37/04

[52] U.S. Cl. ..... 260/122; 260/112 B; 424/101; 424/177

[58] Field of Search ..... 260/112 B, 122; 424/101, 177

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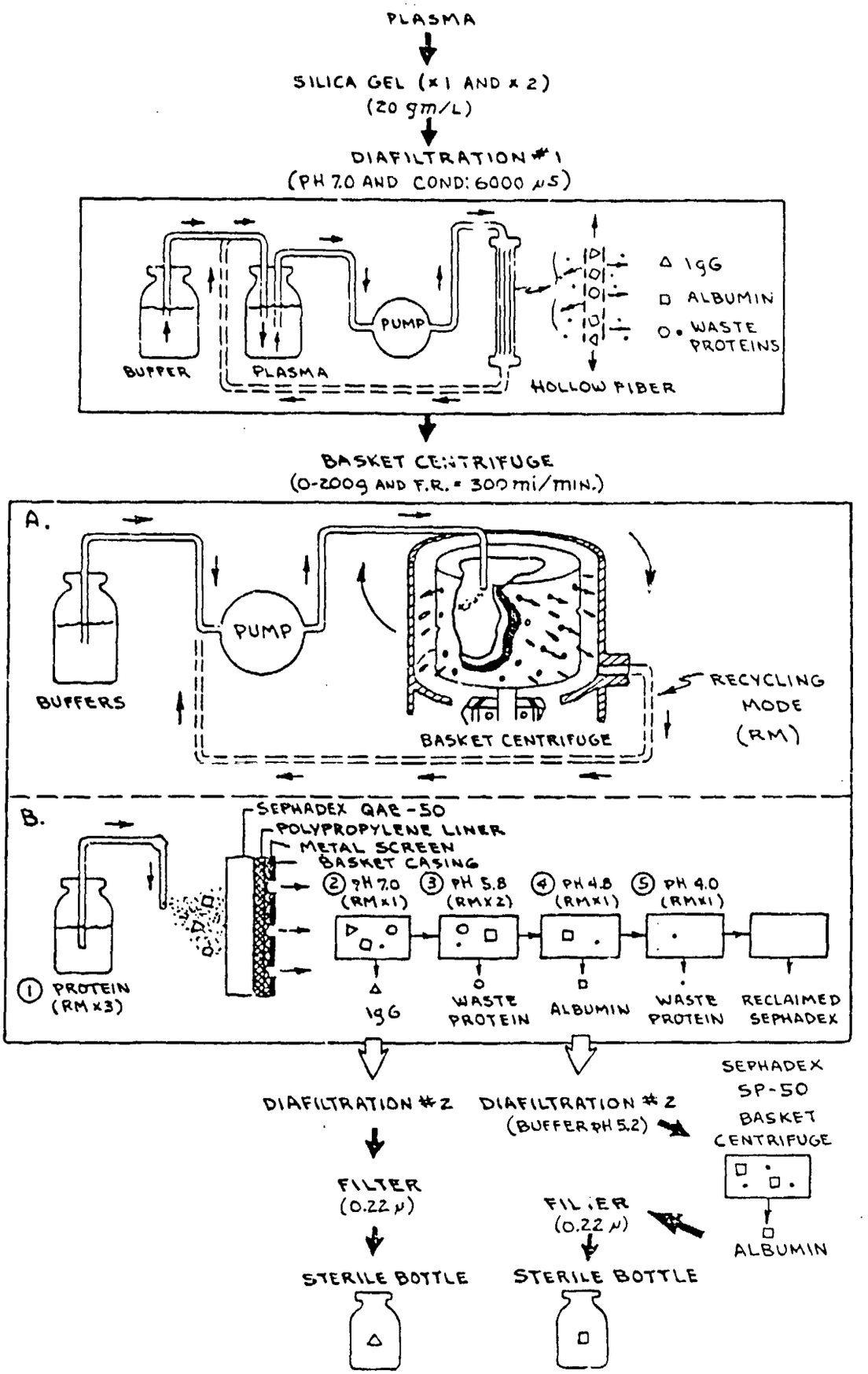
[57] ABSTRACT

A method of isolating and purifying natural, unaltered, undenatured immune gamma globulin (IgG) for intravenous administration and albumin from animal blood plasma, especially human, and the resulting products. The method involves the initial stabilization of plasma by treatment with silica, or the use of previously stabilized plasma. IgG and albumin are isolated from the stabilized plasma by chromatographic reaction with sterile ion exchange resin and eluted by adjustment of pH and ionic strength. The products are concentrated, purified further and packaged. They are characterized by high yield and high purity. They are unfragmented and unaggregated, i.e., natural preparation.

15 Claims, 1 Drawing Figure


PLASMA
SILICA GEL (x1 AND x2)
(20 gm/L)
DIAFILTRATION #1
(PH 7.0 AND COND: 6000 μS)
BUFFER
PLASMA
PUMP
IgG
ALBUMIN
WASTE PROTEINS
HOLLOW FIBER
BASKET CENTRIFUGE
(0-200g AND F.R. = 300 ml/min.)
A.
BUFFERS
PUMP
BASKET CENTRIFUGE
RECYCLING MODE (RM)
B.
SEPHADEX QAE-50
POLYPROPYLENE LINER
METAL SCREEN
BASKET CASING
② pH 7.0 (RMx1)
③ pH 5.8 (RMx2)
④ pH 4.8 (RMx1)
⑤ pH 4.0 (RMx1)
① PROTEIN (RMx3)
IgG
WASTE PROTEIN
ALBUMIN
WASTE PROTEIN
RECLAIMED SEPHADEX
DIAFILTRATION #2
DIAFILTRATION #2 (BUFFER PH 5.2)
SEPHADEX SP-50 BASKET CENTRIFUGE
ALBUMIN
FILTER (0.22 μ)
FILTER (0.22 μ)
STERILE BOTTLE
STERILE BOTTLE

PREPARATION OF INTRAVENOUS HUMAN AND ANIMAL GAMMA GLOBULINS AND ISOLATION OF ALBUMIN

The invention described herein was made in part in the course of work under a grant or award from the Department of Health, Education and Welfare.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to a process for the fractionation of animal blood plasma and the isolation and purification of natura unaltered, undenatured immune gamma globulin and albumin. More particularly, the invention relates to the preparation of intravenously injectable human immune gamma globulin.

The plasma fractionation industry in this country and Europe currently uses the method of cold ethanol fractionation developed by Dr. E. J. Cohn of Harvard University in the 1940's. Even though new methods of protein separation have evolved in the ensuing 35 years, the industry has neither chosen to gamble on new methods nor face up to any regulatory inhibition that a new product faces. As a result of this lack of new spirit, no methods have been developed to produce a new large scale system that would lead to a cheaper and better product. It has been demonstrated that there are severe limitations to the Cohn method, the major being that the process does indeed denature some of the proteins which it attempts to purify and isolate.

Several years have been spent in the development of methods and technology for the preparation and purification of animal (horse, goat and rabbit plasma with antibodies against human lymphocytes) plasma. This anti-lymphocyte globulin, a biologic immunosuppressive agent termed ALG, has been demonstrated to have potent immunosuppressive activity against cell mediated immunity, the type of immunity that causes grafts to be rejected.

After demonstrating the efficacy of this material in animals, it was sought to bring this to the clinic as an adjunctive immunosuppressive agent in the management of the human renal transplant patients. At the time of clinical application of this material, it was felt that the following criteria had to be established: safety of the preparation, a method for fractionating and isolating maximal activity in plasma and a method of fractionation that would allow administration of this material to patients by the most effective route. Animal experiments had demonstrated that the administration of the same quantity of IgG by the intravenous route was two to three fold more effective than the intramuscular or other route. In addition, only limited quantities can be administered intramuscularly whereas 10 to 100 times as much can be administered intravenously. Therefore it was sought to produce a preparation of horse anti-lymphocyte globulin that could be administered safely by the intravenous route. This was done with full knowledge that human gamma globulin could not be administered by the intravenous route.

Early studies by Good at Minnesota, Janeway at Harvard, and others, had demonstrated that the intravenous administration of the Cohn fractionated human gamma globulin often produced severe systemic reactions. The cause of these reactions was related to the high concentration of aggregated material due to the presence of a protein denaturing agent found in the fraction removed by the present invention. As a result,

the only approved method for fractionation is the Cohn cold ethanol process and the only approved route of administration of gamma globulin today is the intramuscular route.

Since intravenous administration of fresh plasma produces no reaction, it has been accepted that the aggregated materials were there as a result of the denaturation of the gamma globulin by the alcohol method and, therefore, alternative methods were sought for the purification and preparation of the horse globulin materials. That this has been successful, and that the goal has been achieved of preparing a safe globulin that can be administered intravenously in patients, is supported by the following facts. Up to the present, over 30,000 grams of purified horse anti-lymphocyte globulin have been prepared. This material has been administered intravenously to over 900 renal transplant patients. The total experience with intravenous administrations approaches over 13,000 separate administrations. These intravenous administrations have not been associated with any evidence of reaction at the time of administration. It therefore was felt that the methods utilized in the preparation and isolation of these horse, goat and rabbit gamma globulins could resolve the problems associated with the attempts to produce and prepare from human plasma an intravenous gamma globulin for use in treating life threatening viral and bacterial infections.

Since viral and bacterial infections constitute the leading cause of patient death in renal transplantation, methods were sought that could be used to treat these severe life threatening infections. One attractive approach was the use of a human gamma globulin that could be administered intravenously. Knowing that none of the commercial preparations could be used in this manner, the possibility was explored of fractionating human gamma globulin from plasma according to the methods developed for the fractionation of the animal proteins. It was found in general principle that this could be done. In clinical studies with this new IV human gamma globulin, doses have been administered intravenously ranging from 20 mg/kg/day to 200 mg/kg/day over a 14 day period. Studies to date show promise of establishing efficacy of the intravenous administration of gamma globulin isolated from pooled human plasma in the treatment of life threatening viral and bacterial infections in immunologically compromised patients.

2. Description of the Prior Art

Although there have been numerous attempts to produce an intravenously injectable gamma globulin, none to our knowledge has produced a natural, unaltered and undenatured product. These efforts are reviewed in Stephan (Biotest) U.S. Pat. No. 3,916,026 and Pappenhagen et al (Cutter) U.S. Pat. No. 3,903,262. The Stephan patent discloses the preparation of intravenously injectable non-complement binding gamma globulin by treating complement binding gamma globulin with beta-propiolactone. The Pappenhagen et al patent discloses preparing intravenously injectable modified immune serum globulin by cleaving at least one interchain disulfide linkage of intact immune serum globulin chains having intact intrachain disulfide linkages and replacing the cleaved disulfide linkage with a pair of alkylate mercapto groups.

SUMMARY OF THE INVENTION

The methods and technologies of the present invention are characterized by 1) their extreme simplicity, 2) the speed and rapidity that each step can be performed, 3) the lack of rigid temperature control required since most of the steps can be carried out at room temperature, and 4) the fact that the method can be scaled up to infinitely large industrial scale volumes at a fraction of the current Cohn costs. In addition, the products are "native", natural, undenatured, aggregate-free, sterile, free from virus and can be isolated in higher yields than with the alcohol fractionation method and in a purer state.

The method involves three basic manipulations. The first step: plasma stabilization, the second: isolation and elution from ion exchange resins of gamma globulin and albumin, and the third step: concentration, dialysis and sterile filtration. The plasma stabilization step comprises treatment with fumed colloidal silica by admixing plasma with silica and then separating stabilized plasma from the silica with adsorbed constituents. The stabilization requires one hour to complete and accomplishes removal of a number of aggregable and easily denaturable plasma proteins. Also removed are the hepatitis associated antigen in plasma and a number of proteolytic enzymes and their precursors. The presence of these enzymes otherwise can lead to the degradation and aggregation of other plasma proteins and the activation of the kinin system. The stabilized plasma is then ready for the next step or can be filter-sterilized and stored at room temperature for periods exceeding two years. Storage of stabilized plasma at room temperature for periods exceeding two years does not alter its biological support properties and no increases in turbidity or precipitation have been observed.

The second step involves the isolation of the IgG and albumin from the stabilized plasma by reacting this material with a sterile ion exchange resin. The IgG and albumin are eluted by adjustments of pH and ionic strength. In addition, pyrogenic activity of plasma is removed by these resins. This ion exchange separation step requires approximately 40 minutes and results in the isolation of an undenatured, monomeric (molecular weight 160,000) aggregate free human IgG 99% pure, with yields of between 60–70%. The I.V. IgG contains less than 1% aggregates and less than 1% dissociated materials. The albumin can then be recovered with over 97% purity and with yields of between 80–90%, compared to the less than 50% yields of the Cohn method. The final step involves the concentration, dialysis, and sterile filtration of these plasma proteins. This final step requires between two to three hours for completion.

As illustrated by the examples, the process is readily scaled up for large capacity production. Although processing of lots of 4 liters and 100 liters are described in detail, lots of 1000 liters or more can readily be processed on existing equipment.

The final products have been subjected to the standard quality control tests that have been set forth by the Bureau of Biologics. These tests include testing for sterility, pyrogenic activity, and toxicity. In addition, tests for aggregation, deaggregation and molecular weight have been performed. Finally, these materials have been tested for and shown to be free of hepatitis associated antigen, by radio immunoassay. Intravenous administration of large quantities (over 30 grams) in over 50 patients has shown no evidence of passage of hepatitis virus nor produced cases of hepatitis.

BRIEF DESCRIPTION OF THE DRAWING

The invention is illustrated schematically, in flow sheet form, in the accompanying drawing.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Blood plasma or plasma fractions containing gamma globulin and/or albumin are stabilized by treatment with fumed silica (colloidal synthetic silicon dioxide of highest quality containing siloxan and silanol groups on its surface) according to the methods described in detail in Condie et al U.S. Pat. No. 3,998,946 or Stephan U.S. Pat. No. 3,686,395. The disclosures of these patents are incorporated herein by reference. Fresh or fresh frozen human or other animal blood plasma or outdated and/or cryoprecipitate human or other animal plasma may be used. The plasma product is intimately admixed with fumed silica, in either wet or dry form, followed by separation of the silica with its adsorbed lipoproteins, cholesterol, triglycerides, fibrinogen and plasminogen-plasmin enzyme system. The resulting stabilized purified plasma product may be stored or can immediately be treated to isolate the gamma globulin and albumin.

To increase yields, it is desirable to resuspend the separated silica, to recover trapped protein from the silica. The material is centrifuged and the supernatant is collected. The precipitate is desirably washed and the wash liquid and the recovered supernatant are added to the product from the silica treatment. The silica treated material is adjusted to the correct pH (about 7 ± 0.2), conductivity (about 6.0 ± 1.0 at 22° C) and volume by concentration and diafiltration or dialysis against an imidazole-acetate buffer (pH 7.0) in preparation for ion exchange treatment.

Although the gamma globulin and albumin may be isolated from the silica treated stabilized plasma product by column chromatography, it is preferred to use a basket centrifuge for its greatly increased flow rates. A variable speed centrifuge is used with speed settings for application of G forces from about 10 to about 250.

The ion exchange separation utilizes a strongly basic anion exchanger such as a sterile pre-swelled modified dextran with quaternary aminoethyl functional groups to separate the gamma globulin. An exemplary material is Sephadex QAE-50, composed of dextran chains cross-linked to give a three-dimensional polysaccharide network with quaternary aminoethyl groups attached by ether linkages to the glucose units of the dextran chains. The silica treated plasma is applied to the exchanger bed and recycled. pH 7.0 imidazole-acetate buffer is applied and pooled with IgG fraction.

Aliquots of pH 5.8 (± 0.1) imidazole-acetate buffer are applied, recycled and discarded. Then aliquots of pH 4.8 (± 0.1) imidazole-acetate buffer are applied to the QAE bed and recycled to separate the albumin fraction. After elution of the albumin from the initial ion exchanger, the albumin is purified by use of a further ion exchange material, a strongly acid cation exchanger such as a sterile modified dextran with sulphopropyl functional groups. An exemplary material, available as Sephadex SP-50, is composed of dextran chains cross-linked to give a three-dimensional polysaccharide network with sulphopropyl groups attached by ether linkages to the glucose units of the dextran chains.

The IgG and albumin pools are treated separately. The IgG is subjected to diafiltration against a glycine-saline buffer and, after sterile filtration, is bottled.

The albumin from the QAE chromatography is subjected to diafiltration against pH 5.2 (± 0.1) imidazole-acetate buffer and then to sulphopropyl chromatography. Although a column may be used, a basket centrifuge is preferred. The concentrated and purified albumin is subjected to final diafiltration against glycine-saline buffer and, after sterile filtration, is bottled.

EXAMPLE 1

The invention is further illustrated in the following detailed example of the preparation of human IgG and albumin:

1. Collection, Storage and Pooling of Human Plasma.

Both outdated and/or cryoprecipitate human plasma is obtained and is stored at −20° C until it is to be used. Only hepatitis antigen tested plasma is used. The plasma is thawed at room temperature, and pooled, but not allowed to reach room temperature. Properties: Volume = 4.0L.; Conductivity = 11.4 millisiemens at 22° C (11.1–11.8); Protein Concentration = 59.2±2.8 mg/ml; pH = 7.50 (7.43–7.86); Time = 5–10 minutes (not including the time it takes to thaw the plasma).

Volumes: (Starting 4.0 liters)	% of starting plasma
T.P. = 236.8 gm. ± 11.2	100
Alb. = 157.6 gm. ± 18.8	100
IgG = 37.2 gm. ± 4.4	100

2. Stabilization of Plasma: Removal of Fibrinogen, Plasminogen, Plasmin, Denatured Immunoglobulins and Lipoproteins Using SiO_2

Dry SiO_2 (Aerosil 380, Degussa, Inc., New York) is added to the plasma as it is stirred using a propeller stirrer until the final SiO_2 concentration is 20 g/L. Although stirring is continued for 1 hour at room temperature after the SiO_2 addition is completed, the reaction between SiO_2 and plasma is essentially completed in less than 20 minutes. The SiO_2 and bound components are removed by centrifugation at 6500 XG for 30 minutes at 0–4° C or by passing through a basket centrifuge or hollow fiber (300,000 or molecular weight pore size). The supernatant fluid is poured off and saved. The precipitate is washed with 0.9% NaCl solution to recover trapped protein. Albumin loss due to trappage in SiO_2 ppt. can be reduced as can IgG loss (See step No. 4). Properties: Volume = 3.4±0.4 L.; Conductivity = 11.8 millisiemens at 22° C (11.3–12.1); Protein Concentration = 54.0±2.2; pH = 7.40 (7.37–7.83); Time = 2 hours.

	% of starting plasma
Volume = 3.4 L ± 0.4	85.5
T.P. = 183.6 ± 7.5	77.5
Alb. = 123.8 ± 8.5	78.6
IgG = 26.2	78.2

3. Second SiO_2 Treatment

The supernatant fluid from step No. 2 is treated a second time with the same amount of SiO_2 (20 g/L. based on the original plasma volume) as in step No. 2. Aerosil and bound components are removed as before. Properties: Volume - 2.92 ± 0.3; Conductivity = 11.6 millisiemens at 22° C (11.2–12.3); Protein Concentration = 46.5 ± 2.6; pH = 7.35 (7.14–7.62), Time = 2 hours.

	% of starting plasma
Volume = 2.92 ± 0.3	72.5
T.P. = 135.8 ± 7.8	57.3
Alb. = 119.0 ± 21.4	75.3
IgG = 16.6 ± 3.5	44.6

4. Recovery of Plasma Proteins from SiO_2 Precipitates to Increase Yields.

To recover trapped protein from the SiO_2 precipitates from steps No. 2 and No. 3, the precipitates are resuspended with 500 ml. of 0.9% NaCl which is 12.5% of the original plasma volume and centrifuged at 6500 XG for 30 minutes at 0–4° C and the supernatant is collected. The precipitates are washed twice and the recovered supernatants are added to the material remaining after the second SiO_2 treatment. Then the process proceeds to diafiltration step No. 5.

SiO_2 Washes:		SiO_2 #1 Washes		SiO_2 #2 Washes	
	Recovery	% Starting Plasma		Recovery	% Starting Plasma
Volume	925 ml	23		800 ml	20
T.P.	21.3 g.	9		27.5 g.	12
Alb.	17.3	11		17.2	11
IgG	5.2	14		3.9	10
SiO_2 Washes plus Post SiO_2 #2			% starting plasma		
Volume	4.64 L.		116		
T.P.	184.6 g.		78		
Alb.	154		98		
IgG	25.7		69		

5. Diafiltration #1 Against pH 7.0 Imidazole-acetate buffer (#1)

The SiO_2 treated material is adjusted to the correct pH, conductivity and volume by concentration and diafiltration (or dialysis) in a Millipore Pellicon Membrane Concentrator (Millipore Corp., Bedford, Mass.) with 15 sq. feet of PTGC membrane (10,000 daltons cutoff) or another similar device using membranes with a molecular weight cutoff of 10,000 to 50,000 daltons. Diafiltration is accomplished by adding sterile pH 7.0 imidazole-acetate buffer (#1) at the same rate as filtrate is removed. Diafiltration is completed when the sample has reached pH 7.0 ± 0.05 at 22° C and conductivity is 6.0 ± 0.2 millisiemens at 22° C. Properties: Volume = 3.7 L.; Conductivity = 6.0 ± 0.2 at 22° C.; Protein Concentration = 50 mg/ml; g/g QAE = 0.94; pH = 7.0 ± 0.05; Time = 1 hour.

		% starting plasma
Volume	= 3.7 L.	116
T.P.	= 184.6 g.	78
Alb.	= 154 g.	98
IgG	= 25.7 g.	69

6. QAE-50 Chromatography (at 22° C).

A Western States STM-1000 basket centrifuge with a 12" × 5" basket lined with a 1 micron polypropylene liner is loaded with 197.2 g. Sephadex QAE-50 (pre-

swelled in pH 7.0 imidazone-acetate buffer (#1). After the bed has been packed for ≃ minutes at 220 XG (1140 RPM), the speed of the centrifuge is reduced to 52 XG (550 RPM) and run another ≃5 minutes. The same buffer (#1) applied to bed through a Flood Jet ¼ K1.5 nozzle at ≃0.3 L./minutes throughout packing procedure.

The sample (3.7 L. at 50.0 mg/ml, total protein — 185 gm) is now applied at 0.3 L./min and recycled twice. Then, 12.0 L. of the same pH 7.0 imidazole-acetate buffer (#1) are applied and pooled with the sample. This is the IgG fraction, (≃0 g at 1.6 mg/ml).

Then 4–4L. aliquots of pH 5.8 imidazole acetate buffer (#3) are applied — each aliquot is recycled once. This pool is discarded.

Next 4–4 L. aliquots of pH 4.8 imidazole-acetate buffer (#4) are applied — the first aliquot is recycled once. This 16 L. pool is the albumin fraction (126 g. at 7.9 mg/ml). If the gel is to be recycled for further use, 12 L. of pH 4.0 sodium acetate buffer (#2) is applied and discarded. Time = 4.5 - 5.0 hours. The basket centrifuge is run at 50 XG. Lower G forces appear to be desirable since larger quantities of protein/gm. of Sephadex can be applied without the breakthrough of contaminants.

	IgG Pool		Albumin Pool	
Volume	15.7	L.	16.0	L.
Total protein	19.7	g.	156.8	g.
Protein conc.	1.25	g/L	10.7	g/L
pH	7.0		=4.9	
Conductivity	6.2	mS	6.2	mS
Flow rate	0.3	L/min.	0.3	L/min.
G/G QAE	0.10		0.80	
% of starting plasma	53.0	(of IgG)	88.6	(of Alb.)

The IgG and albumin pools are then treated separately: IgG to step #7; Albumin to step #9.

7. IgG Diafiltration #2 (at 4° C against glycine-saline buffer (#5) for IgG

Final Values	Volume	Protein	pH	Cond.	Time	Flow Rate
IgG						
Pool	394 ml.	50 mg/ml	6.8±0.05	13 mS	1 hr.	350 ml/min

8. (filter sterilely with 0.20 μ Pall Ultipor filter and dispense into 40 ml vials). Time = 1 hour.

Final IgG properties: pH 6.8; Conductivity 13 mS; Protein concentration 50 g/l; no measurable impurities. The product passed LAL, pyrogen and safety tests.

9. Albumin

Post QAE Albumin Pool:

Albumin	89.1
α2macroglobulin - 3.7%	2.5
IgA - 2.3%	1.6
IgM - 1.7%	1.3
α1 antitrypsin - 1.6%	2.1
Others - 3.6%	3.4

10. Albumin Diafiltration #2 (at 4° C against pH 5.2 imidazole-acetate buffer (#6).

Final Values:

Volume	= 3.14 L.
Protein	= 50 mg/ml
pH	= 5.20 ± 0.05
Cond.	= 5500 mS
Time	= 1 hour
Flow rate	= 350 ml/min

11. SP-50 Chromatography (at 22° C).

The sample is passed through Sephadex SP-50 (preswelled) in pH 5.2 imidazole-acetate buffer (#6). Basket centrifuge as described in step #6 or column may be used. Albumin Yield = 100% on this step.

12. Albumin Diafiltration #3 (at 4° C against glycine-saline buffer (#5).

Final Values:

Volume	= 3.14 L.
Protein	= 50 mg/ml
pH	= 6.80 ± 0.05
Conductivity	= 13 mS
Time	= 2.5 - 3 hours
Flow Rate	= 350 ml/min

13. Bottle (filter sterilely with 0.20 μ Pall Ultipor filter and dispense into 40 ml vials). Time = 1 hour.

Final albumin properties: pH = 6.8; Conductivity = 13 mS; Prot. Conc. = 50 g/L; impurities 2.1%. The product passed LAL, pyrogen and safety tests.

The yields and recoveries are summarized in Table I:

TABLE I

	Plasma	SiO_2 #1	SiO_2 #2	SiO_2 & Washes	QAE	SP
Volume	4.0	3.4±0.4	2.92±0.3	4.64	—	—
% volume	100	85.5	72.5	116		
Total protein	2.36±11.2	183.6±7.5	135.8±5.8	184.6	--	—
% T.P.	100	77.5	57.3	78		
Albumin	157.6±18.8	123.8±8.5	119.0±21.4	154.0	139.7	139.
% albumin	100	78.6	75.3	98	88.6	88.
IgG	37.2±4.4	26.2±3.7	16.6±3.5	25.7	19.7	—
% IgG	100	78.2	44.6	69	53.0	
Prot. Conc.	59.2±2.8	54.0±2.2	46.5±2.0	29.8	—	—

The composition and properties of the buffers are set forth in Table II:

TABLE II

1. pH 7.0 Imidazole-acetate
48.0 g. Imidazole
610.0 g. sodium acetate
80.1 ml 6.0M acetic acid
15 gal. N.P. H_2O
Cond = 6.15 ± 0.1 mS at 22° C
pH = 7.0 ± 0.05

2. pH 4.0 Sodium-acetate
852.0 g. sodium-acetate
1220.0 ml glacial acetic acid
15 gal. N.P. H_2O
Cond = 6850 ± 0.1 mS

TABLE II-continued

pH = 4.0 ± 0.05
3. pH 5.8 Imidazole acetate
7.0 imidazole-acetate (#1)
S pH'ed with 4.0 sodium-acetate (#2)
(≈4.5 L. pH 4) to 15 gal. of #1
4. pH 4.8 Imidazole-acetate
7.0 imidazole-acetate (#1)
pH'ed with 4.0 sodium-acetate (#2)
(≈9.0 L. pH 4) to 15 gal. of #1
5. Glycine-saline
1265 g glycine
498.8 g. NaCl
15.0 gal. N.P. H_2O
adjust pH with 5N NaOH to 6.80 ± 0.05
(less 100 ml NaOH)
6. pH 5.2 Imidazole-Acetate Buffer-Full Capacity -5500 cond
S 22.33 g. imidazole
169.4 g. NaAc
51.0 ml. glacial HAc
18 L N.P. H_2O (to bottom of cap)
pH = 5.20 ± 0.05 Conductivity = 5500 μS ± 100 at 22° C

EXAMPLE 2

Large volume preparation of human intravenous gamma globulin is illustrated by the following:

1. Collection of human plasma from donors

Plasma is collected from healthy, volunteer adult donors. Aseptic techniques are utilized in the collection of this sterile plasma. It is either cryoprecipitated or outdated, is frozen at −20° to −70° C and stored sterilely in a closed storage environment. Individual records are kept on the cryoprecipitated plasma sources. It is not possible to always keep records on the pooled outdated plasma. However, only plasma that has been tested for hepatitis B associated antigen is used.

2. Preparation of pooled human plasma for protein fractionation

Preparation of the crude human plasma for fractionation of the gamma globulin is divided into the following steps: 1) prepooling safety testing, 2) pooling, (100 to 130 liter pools). Each lot of plasma is double checked to insure that it contains no hepatitis B-associated antigen. Then approximately 400–500 blood bags containing the human plasma are assembled, thawed, and pooled into a 220 liter sterile container. Samples are taken at this stage to test for hepatitis B-associated antigen, and then against appropriate viruses, bacteria and fungi, and against A and B human blood group substances. A total protein, gamma globulin concentration, albumin concentration, pH, and conductivity of the pool is determined.

One hundred liters are warmed at room temperature until thawed. However, the bags are immediately chilled at 4° C so they never reach room temperature. The bags are then immersed in 70% ethanol solution and the excess solution is drained off. Each bag is cut open and the contents poured fast into a sterile graduated cylinder, the volume recorded, and then poured into a sterile 220 liter polypropylene container. The pool is mixed and a sample removed for testing and sterility. The container of plasma is stored at 4° C overnight and then fractionated.

3. Fractionation of the crude pooled plasma

The pooled human plasma is fractionated according to the following outlined procedure:

A. Stabilization of the plasma by the addition of synthetic sterile pyrogen free silicon dioxide

Dried, synthetic, sterile silicon dioxide (Aerosil 380) is added to a final concentration of 40 grams/liter of the pooled plasma. The plasma and silicon dioxide are mixed with the aid of motor driven rotors, care being taken to avoid foaming which can result in denaturation. This mixing is continued for one hour at room temperature. Following mixing, the silicon dioxide and its absorbed plasma proteins are placed in either a Beckman J6 centrifuge or a RC3 centrifuge for a thirty minute period and sedimented at 6,000 X G. Stabilization involved the removal of fibrinogen and any split products, the removal of the easily denaturable lipo-proteins and associated HLA antigens, and finally the removal of the plasminogen-plasmin, a proteolytic enzyme system which has been implicated in IgG aggregate formation and the partial degradation of gamma globulin. The supernate from the low speed centrifugation is pooled and run through a high speed continuous flow rotor at 32,800 X G to remove any other aggregated materials. This step is accomplished at +4° C at a rate of 350 ml/minute. The rotor is sterilized with 70% ethanol and rinsed with non-pyrogenic water prior to use. A post HSS sample is removed for biuret protein concentration determination.

B. Dialysis and concentration of the post silicon dioxide stabilized treated plasma pool

Following the high speed spin, the protein concentration of the sample is adjusted to 50 g/l and diafiltrated with pH 7.0 imidazole acetate buffer (pH = 7.00 ± 0.05 and conductivity = 6.0 ± 0.1 mS at 22° C). One of two methods may be used:

i. Using an Amicon DC-30 unit with three cartridges with surface areas each 10 sq. ft. with a 50,000 MW cutoff, the pool is concentrated to about 50 g/l. Diafiltration is then accomplished by adding the imidazole-acetate buffer through a sterile 0.20 micron Pall Ultipor DFA 3001 ARP filter (a non-asbestos, non-glass fiber containing filter) to the pool as the concentration continues, so that the volume remains constant. The operation is concluded when the sample has a pH of 7.0 ± 0.05 and conductivity of 6.0 ± 0.1 mS. This is usually accomplished after 3–4 volumes of buffer have been added. The DC-30 is cleaned with a 0.1 N NaOH and 0.05% sodium hypochlorite, rinsed with a large volume of non-pyrogenic H_2O and stored in 25% ethanol.

ii. This method is similar to method i but uses a Millipore Pellicon Apparatus equipped with 25 sq. ft of membrane with a 10,000 MW cut-off. The same procedure is followed and the Pellicon is cleaned with 0.1 NaOH, then 5% acetic acid, rinsed with non-pyrogenic H_2O and stored in 25% ethyl alcohol.

In both methods i and ii samples of the pool and the filtrate are collected for biuret protein concentration determination.

C. Sterile filtration of the pool through 0.22 micron Millipore of Pall filters as an intermediate sterilization step in the procedure

D. Fractionation of human gamma globulin

i. QAE Sephadex columns

Anion exchange chromatography is done using four 16 l bed capacity stacks containing about 650 g each Sephadex QAE A50 gel swelled in 2.87 mS pH 7.0 ±

0.05 imidazole-acetate buffer containing 25% ethanol. The stacks and all the tubing are sterilized by leaving them in this buffer for at least 24 hours. The buffer is washed out just prior to use with starting buffer filtered through a sterile 0.2 micron Pall filter. The stacks are arranged in parallel flow and the flow rate is kept constant at 150 ml/min/stack using gravity as the driving force. The sample, previously diafiltrated to meet specified conditions of pH 7.0 ± 0.05 and conductivity of 6.0 ± 0.1 mS at 22° C is spun at 6,500 X G for 30 min. at 22° C and then applied to the top of the stacks and samples are collected in sterile 4.0 l graduated cylinders. The column is monitored by following the absorbance at 280 nm of the protein peak. After ½ of the sample has been applied, the rest of the protein unbound to the gel is washed out using the sterile pH 7.0 imidazole-acetate buffer. The protein is pooled into a sterile polypropylene container and stored at 4° C until the second half of the sample can be added to it. The stacks are recycled by running at 2 bed volumes of pH 5.0 0.1 ionic strength acetate buffer, 2 bed volumes of pH 4.0 0.1 ionic strength acetate buffer, and 3 bed volumes of pH 7.0 0.1 ionic strength imidazole-acetate buffer. All buffers are made up of sterile pyrogen deionized distilled water and sterile filtered just prior to use. The second half of the sample is then spun, applied to the column and eluted in the same manner as the first half. The pH 7 protein peaks from both runs are pooled and treated further and the stacks are dismantled and repacked using new resins.

ii. High G force basket centrifuge QAE chromotography

A variable speed basket centrifuge equipped with a one hundred l. capacity is lined with a one micron polypropylene liner and loaded with about 3950 g of QAE-50 Sephadex. The liner, rotor, tubing, and all parts are sterilized either by autoclaving or soaking in 70% ethanol prior to use. The Sephadex is pre-swelled and sterilized in pH 7.0 ± 0.05 and 0.1 ionic strength imidazole-acetate buffer containing 25% ethanol. After the Sephadex bed has been packed for about five minutes at 220 X G (1140 RPM), the speed of the motor is reduced to 52 X G (550 RPM) and run another five minutes. Imidazole-acetate buffer without ethanol is applied to the bed through a Flood Jet ¼ KSS 35 at 7 pounds pressure or ¼ K15 stainless steel nozzle at about 7.5 l/min throughout the packing procedure. After the bed is equilibrated with the buffer, 50 l of sample are applied and recycled twice (8 to 10 minutes each — total 20 minutes). Then imidazole-acetate buffer is flushed through the gel and added to the sample. The Sephadex is reclaimed by applying ph 4.0 and 0.1 ionic strength sodium-acetate to the bed to remove waste proteins. The bed is reequilibrated as above, and the next 50 l of sample may be applied as above. The protein from both runs is pooled, mixed, and a sample removed for biuret protein concentration determination. It is then diafiltrated against glycine saline buffer (pH = 6.80 ± 0.05 and conductivity = 13 mS) until the pH = 6.80 ± 0.05 and the conductivity = 12 - 15 mS. Samples of the pool and filtrates are collected for biuret protein concentration determination. Total time is less than one hour.

E. High speed spin

The pool is now high-speed spun in a Beckman J-21B centrifuge with a JCF-2 flow through rotor at 32,800 X G and 4° C at a flow rate of 350 ml/min. The rotor is sterilized with 70% ethanol and rinsed with non-pyrogenic H_2O prior to use. A sample is removed for biuret protein concentration determination.

F. Sterile filtration

Immediately following the high-speed spin, the pool is prefiltered in preparation for bottling through a 0.20 micron Pall Ultipor DFA 3001 AKP filter (a non-asbestos, non-glass fiber containing filter) which has been autoclaved at 132° C for one hour. It is then final filtered through another sterile .20 micron Pall filter and then through a Millipore 0.45 micron HAWP 14250 membrane filter which has been autoclaved at 121 degrees for 20 minutes. Sterile technique is carefully followed during all stages of this operation. The 0.45 micron membrane filter is sterilely transferred to and cultured in 1000 ml of TSB culture media for 7 days. A sample of this culture is assayed for sterility on day 7.

4. Bottling, storage, and packaging

The intravenous immune serum globulin (human) is sterile filled into 40 ml vials which have been previously washed in non-pyrogenic distilled water and steam autoclaved for sterility. Sterilization of the immune serum globulin (human) is obtained either by Millipore filtration using a sterilized .45 micron prefilter and a 0.22 micron final filter or by a Pall filter system starting with a .45 micron prefilter and a 0.22 micron final filter. The filtered intravenous immune serum globulin (human) is collected and automatically pipetted into 40 ml bottles. These are sterilely capped and sealed. The bottled material is labeled, and stored for 2 weeks at either −20° C or 3 to 5° C until all tests have been completed. If all tests are passed, it is approved for administration.

The multiple injection 40 ml vials are individually labeled with proper label, lot number, and expiration date according to Section 73.50 Public Health Service regulations. They are placed in stainless steel locked boxed for refrigeration at −20° C or 3° to 5° C. When removed for distribution, the labeled vial is individually packed in a labeled box with lot number, expiration date, and Information Leaflet according to Section 73.52 Public Health Service regulations.

The IgG product is fully capable of intravenous administration without adverse reaction or effect. The advantages to be gained by the use of an intravenous human IgG that has not been denatured by preparation, or altered by chemical or enzymatic processes include the following: 1) prolonged half-life in the circulation, 2) higher maximal levels following intravenous administration, 3) maximal therapeutic activity, 4) no systemic reactions, and 5) painless route of administration allowing for 10 to 50 fold increase in dosage compared with other routes. Intravenous human IgG prepared by the described methods has been tested clinically in doses ranging from 20 mg/kg/day for ten days to 200 mg/kg/day for six days. The purpose in using the higher dose range was to achieve complete replacement of the total body IgG pool. In clinical trials, this high dose treatment schedule resulted in 11 of 13 patients with CMV interstitial-pneumonitus making complete recoveries. This is noteworthy, since past experience with this disease indicated that it was uniformly fatal. The total experience is with over 500 intravenous injections of this preparation with no undesirable side effects or reactions. Studies are proposed to use hyper-immune intravenous IgG, for not only prophylaxis but therapy

of a number of viral and bacterial life threatening infections.

Albumin is isolated in significantly greater yields and purity than with the alcohol method. The described process makes possible a superior product at markedly reduced costs.

In summary, a new methodology has been developed for human plasma fractionation. The methods and technology can be characterized by 1) their simplicity, 2) the speed that each step can be performed, 3) the lack of rigid temperature requirements, since most of the processes can be carried out at room temperature, 4) the ease in scaling up to large industrial-scale volumes, 5) the removal of pyrogens and hepatitis associated antigen from plasma (at two stages or steps in the procedure), and 7) the isolation of undenatured natural material of highest purity and in significantly greater yields than can be accomplished with the current industrial-scale alcohol procedures.

It is apparent that many modifications and variations of this invention as hereinbefore set forth may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only and the invention is limited only by the terms of the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of isolating and purifying natural immune gamma globulin for intravenous use and albumin from blood plasma, which method comprises:

(A) intimately admixing a human blood plasma product containing gamma globulin and albumin and contaminating blood proteins, lipids, lipoproteins and proteolytic enzymes with finely divided sterile fumed silica,

(B) separating the silica with adsorbed contaminating blood proteins, lipids and lipoproteins from the remaining stabilized plasma product,

(C) adjusting the pH of the stabilized plasma product to about pH 6.8 to 7.2 and the conductivity to about 5 to 7 (at 22° C),

(D) applying the stabilized plasma product to a strongly basic anion exchanger and separating purified gamma globulin therefrom,

(E) eluting the albumin from the anion exchanger by applying a buffer of about pH 4.7 to 4.9 thereto, and

(F) applying the eluted albumin to a strongly acid cation exchanger and separating purified albumin therefrom.

2. A method according to claim 1 wherein the separated gamma globulin is recycled to the anion exchanger at least once.

3. A method according to claim 1 wherein a buffer of about pH 5.7 to 5.9 is applied to the anion exchanger after separation of gamma globulin and before elution of albumin, and discarded.

4. A method according to claim 1 wherein said gamma globulin is further purified by diafiltration and sterile filtration.

5. A method according to claim 1 wherein said albumin is further purified by diafiltration and sterile filtration.

6. A method according to claim 1 wherein said ion exchanger treatments are carried out in a basket centrifuge under high gravity force chromatography.

7. A method according to claim 1 wherein:

(A) said stabilized plasma product is applied to a strongly basic anion exchanger in a basket centrifuge, separated, recycled at least once and reseparated,

(B) a buffer of about pH 5.7 to 5.9 is applied to the anion exchanger and discarded.

(C) said eluted albumin is applied to a strongly acid cation exchanger in a basket centrifuge and separated, and

(D) said gamma globulin and albumin are further and separately purified by diafiltration and sterile filtration.

8. A method of isolating and purifying immune gamma globulin for intravenous use and albumin from a silica stabilized blood plasma product containing gamma globulin and albumin, which method comprises:

(A) adjusting the pH of the stabilized plasma product to about pH 6.8 to 7.2 and the conductivity to about 5 to 7 (at 22° C),

(B) applying the stabilized plasma product to a strongly basic anion exchanger and separating purified gamma globulin therefrom,

(C) eluting the albumin from the anion exchanger by applying a buffer of about pH 4.7 to 4.9 thereto, and

(D) applying the eluted albumin to a strongly acid cation exchanger and separating purified albumin therefrom.

9. A method according to claim 8 wherein the separated gamma globulin is recycled to the anion exchanger at least once.

10. A method according to claim 8 wherein a buffer of about pH 5.7 to 5.9 is applied to the anion exchanger after separation of gamma globulin and before elution of albumin, and discarded.

11. A method according to claim 8 wherein said gamma globulin is further purified by diafiltration and sterile filtration.

12. A method according to claim 8 wherein said albumin is further purified by diafiltration and sterile filtration.

13. A method according to claim 8 wherein said ion exchange treatments are carried out in a basket centrifuge under high gravity force chromatography.

14. A method according to claim 8 wherein:

(A) said stabilized plasma product is applied to a strongly basic anion exchanger in a basket centrifuge, separated, recycled at least once and reseparated,

(B) a buffer of about pH 5.7 to 5.9 is applied to the anion exchanger and discarded,

(C) said eluted albumin is applied to a strongly acid cation exchanger in a basket centrifuge and separated, and

(D) said gamma globulin and albumin are further and separately purified by diafiltration and sterile filtration.

15. A method according to claim 1 wherein said blood plasma product is admixed with finely divided sterile fumed silica in wet form.

* * * * *

United States Patent [19]

Condie

[11] 4,296,027

[45] Oct. 20, 1981

[54] PURE INTRAVENOUS HUMAN AND ANIMAL GAMMA GLOBULINS

[75] Inventor: Richard M. Condie, Minneapolis, Minn.

[73] Assignee: The Regents of the University of Minnesota, Minneapolis, Minn.

[21] Appl. No.: 5,150

[22] Filed: Jan. 22, 1979

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 829,565, Aug. 31, 1977, Pat. No. 4,136,094.

[51] Int. Cl.³ C07G 7/00

[52] U.S. Cl. ..... 260/112 B; 124/101; 124/177

[58] Field of Search ..... 260/112 B; 424/101

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Primary Examiner—Howard E. Schain

Attorney, Agent, or Firm—Burd, Bartz & Gutenkauf

[57] ABSTRACT

Isolated and purified natural, unaltered, undenatured immune gamma globulin (IgG) for intravenous administration prepared from animal blood plasma, especially human. The products are characterized by high yield and high purity. They are unfragmented and unaggregated, i.e., natural preparation.

9 Claims, 2 Drawing Figures

U.S. Patent Oct. 20, 1981 Sheet 1 of 2 4,296,027

FIG. 1

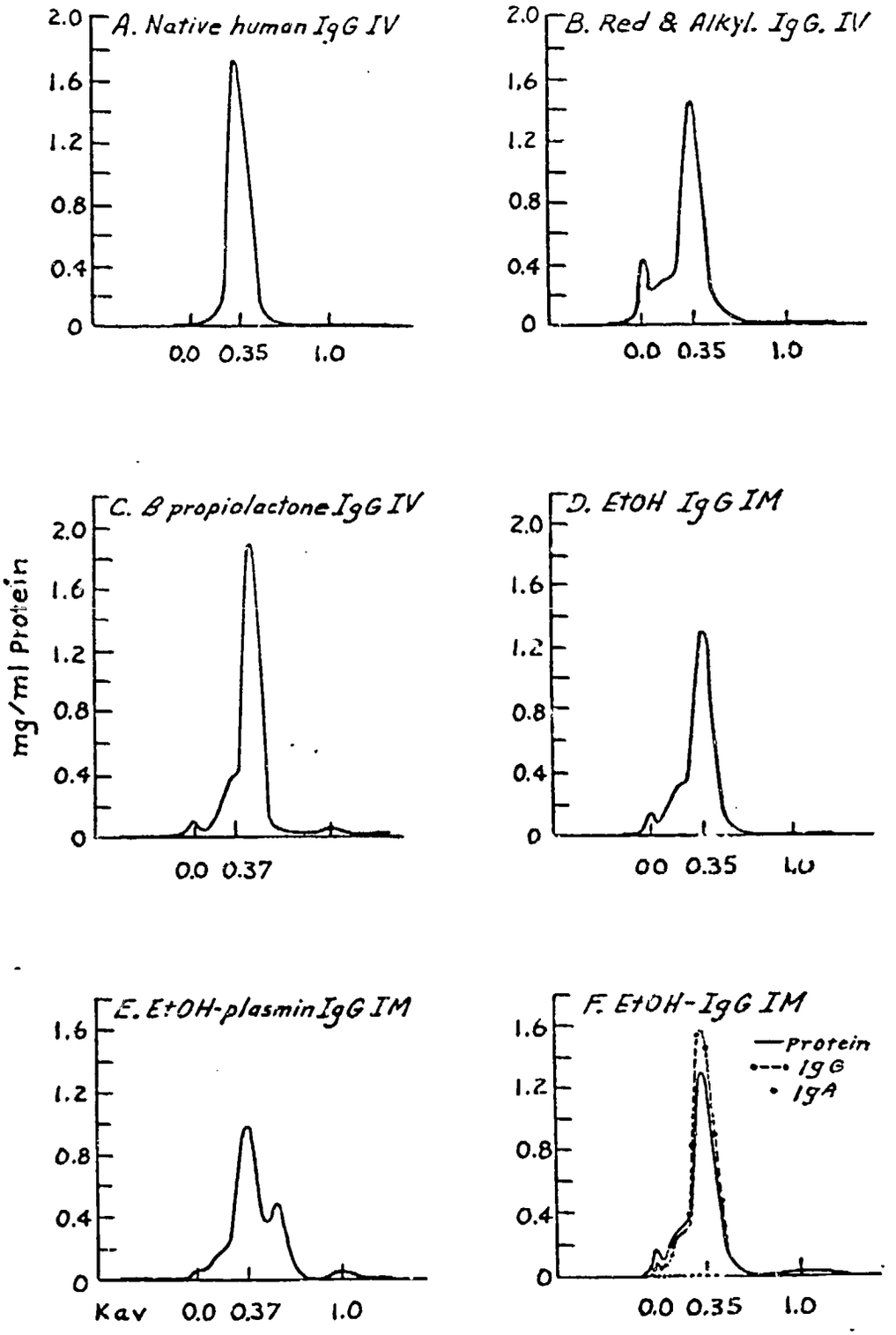
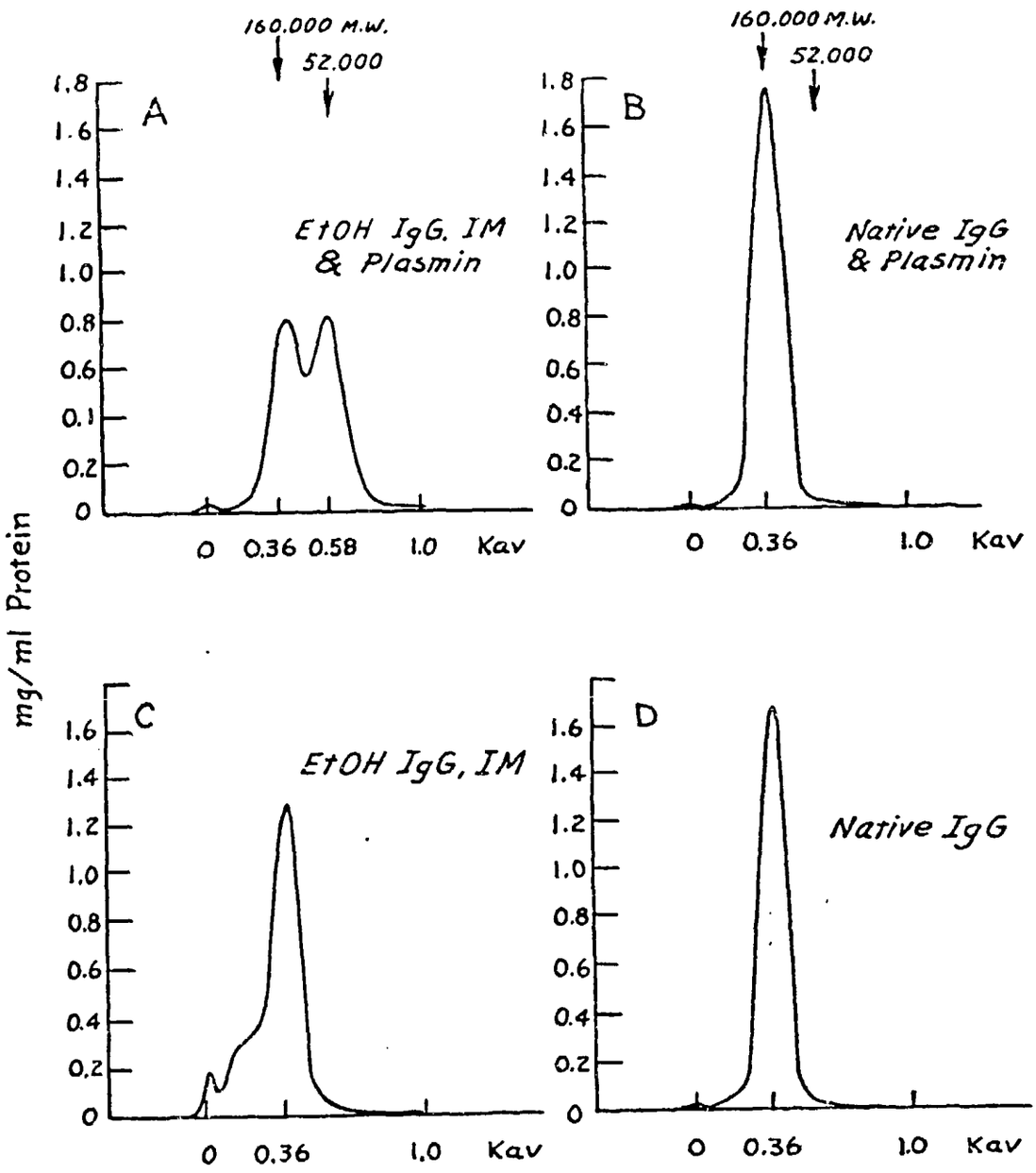


FIG. 2


160,000 M.W.
52,000
A
EtOH IgG. IM & Plasmin
B
Native IgG & Plasmin
C
EtOH IgG, IM
D
Native IgG
mg/ml Protein
Kav
0
0.36
0.58
1.0

PURE INTRAVENOUS HUMAN AND ANIMAL GAMMA GLOBULINS

This application is a continuation-in-part of my prior application Ser. No. 829,565, filed Aug. 31, 1977, now U.S. Pat. No. 4,136,094, issued Jan. 23, 1979.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The invention relates to isolated and purified natural, unaltered, undenatured immune gamma globulin prepared by the fractionation of animal blood plasma. More particularly, the invention relates to intravenously injectable human immune gamma globulin.

SUMMARY OF THE INVENTION

In my aforesaid copending application Ser. No. 829,565, U.S. Pat. No. 4,136,094 whose disclosure is incorporated herein by reference, there is described and claimed a method for producing the intravenous gamma globulins of the present invention. The products are "native", natural, undenatured, aggregate-free, sterile, free from virus and can be isolated in higher yields than with the alcohol fractionation method and in a purer state.

The method involves three basic manipulations. The first step: plasma stabilization, the second: isolation and elution from ion exchange resins of gamma globulin and albumin, and the third step: concentration, dialysis and sterile filtration. The plasma stabilization step comprises treatment with fumed colloidal silica by admixing plasma with silica and then separating stabilized plasma from the silica with adsorbed constituents. The stabilization accomplishes removal of a number of aggregable and easily denaturable plasma proteins. Also removed are the hepatitis associated antigen in plasma and a number of proteolytic enzymes and their precursors, which can lead to the degradation and aggregation of other plasma proteins and the activation of the kinin system.

The second step involves the isolation of the IgG and albumin from the stabilized plasma by reacting this material with a sterile ion exchange resin. The IgG and albumin are eluted by adjustments of pH and ionic strength. In addition, pyrogenic activity of plasma is removed by these resins. This ion exchange separation step results in the isolation of an undenatured, monomeric (molecular weight 160,000) aggregate free human IgG 99% pure, with yields of between 60–70%. The I.V. IgG contains less than 1% aggregates and less than 1% dissociated materials. The final step involves the concentration, dialysis, and sterile filtration of the IgG and albumin.

The final products have been subjected to the standard quality control tests that have been set forth by the Bureau of Biologics. These tests include testing for sterility, pyrogenic activity, and toxicity. In addition, tests for aggregation, deaggregation and molecular weight have been performed. Finally, these materials have been tested for and shown to be free of hepatitis associated antigen, by radio immunoassay. Intravenous administration of large quantities (over 30 grams) in over 50 patients has shown no evidence of passage of hepatitis virus nor produced cases of hepatitis.

Blood plasma of man and mammals contains more than 100 proteins, many of which do not have as yet recognized functions. Some of these proteins are components of multi-enzyme systems such as the complement pathway, blood coagulation, and the fibrolytic and kininogen systems. Other plasma protein systems of great physiologic and medical importance include the immunoglobulins. The immunoglobulins differ from all other plasma proteins in their multiplicity, their heterogeneity, their genetic control, their antibody specificity, and their biologic effector functions. Immunoglobulins are defined as multifunctional proteins that are endowed with both known antibody specificity and biologic effector functions. Further immunoglobulins are endowed with structural features that control and orchestrate a number of critical biologic effector mechanisms through interactions with the complement, fibrinolytic, coagulation, and kininogen system of the blood plasma. Whether these biologic effector functions serve the host or result in harmful systemic reactions is determined by the state of the immunoglobulin molecule. Native immunoglobulins serve the host under normal conditions whereas altered or denatured immunoglobulins result in the generation and triggering of potent, damaging systemic reactions.

Immunoglobulins are formed by the lymphoid cell system of vertebrates and circulate in the blood plasma. They migrate electrophoretically as gamma globulins, but usually are very heterogenous, ranging to the beta globulins. There are five classes of immunoglobulins with the IgG class making up the majority of the circulating immunoglobulins as well as containing the majority of the specific protective antibodies in the immunoglobulins of the plasma.

Isolation and purification of the native IgG protein from the over 100 different proteins in plasma has presented as yet unresolved major obstacles in protein fractionation. These include isolation of the IgG molecule in its native state free of denaturation, and isolation in a highly purified state free of any fragments or activated peptides that have been shown to develop during various fractionation procedures. (Activated peptides have marked pharmacologic effects when injected into man and can produce severe reactions at nanogram levels. Denatured IgG activates the complement pathway, portions of the blood coagulation and kininogen systems resulting in the production of severe systemic reactions.)

CURRENT LICENSED PROCEDURE FOR FRACTIONATION OF HUMAN IgG

The only licensed procedure for the fractionation of human plasma at the present time is the Cohn cold alcohol method. This procedure results in the separation of the three major blood proteins for therapeutic use: albumin—the major regulator of osmotic pressure and plasma; fibrinogen—principle in the blood coagulation system; and gamma globulins—the antibody containing fractions. Fractionation of proteins by this process and others involving protein precipitants or ion exchange chromatography have met with limited success. A significant body of evidence has accumulated which demonstrates that the proteins isolated are not natural, native products, but have been denatured extensively. The Cohn cold ethanol method depends upon balancing the precipitating action of the organic solvent with the solvent actions of the electrolytes present while controlling for five independent variables, namely electrolyte concentration, alcohol concentration, hydrogen ion concentration, temperature, and

protein concentration. To the extent that these variables are controlled depend the purity, yield, and the extent of denaturation of each plasma protein isolated.

Gamma globulins and IgG isolated by these precipitation or ion exchange methods must be administered intramuscularly. Intravenous administration results in a series of undesirable side effects which include erythema, vomiting, abdominal pain, fever, collapse and loss of consciousness. In marked contrast, the native IgG of fresh human plasma may be administered intravenously with no untoward reactions. Therefore, the methods to isolate a native product must produce an IgG that has the same if not similar properties of IgG in fresh plasma.

PRESENT KNOWLEDGE OF THE NATIVE IgG MOLECULE

While extensive, detailed information has been published characterizing the primary, secondary, tertiary, and quaternary structure of the IgG molecule, no documentation exists demonstrating that IgG isolated and utilized in these structural studies retains its biological effector function similar to that of the native molecule of fresh plasma. The standards for characterization of the isolated native IgG molecule must therefore include not only structural details, but comparative evidence of its biologic behavior with reference to the native IgG in fresh plasma.

These biologic and structural parameters include: (1) native electrophoretic range, (2) native IEP (isoelectric point) range, (3) evidence that neither new chemical groupings have been added nor native chemical groups removed or altered, (4) one-half time or turnover time in the circulation of three weeks, (5) low anticomplementary activity, (6) molecular weight of 160,000, (7) no aggregates or low molecular fragments, (8) resistance to limited proteolysis (indicative of a non-denatured native state), (9) [illegible] partial proteolytic damage by activated plasma plasmin, and (10) safe for intravenous injection in large quantities.

Stated differently, intravenous infusion of fresh plasma is safe, the IgG is not anticomplementary, and has a turnover of approximately three to four weeks When plasma is fractionated by the current licensed cold ethanol procedure of E. J. Cohn, by ammonium sulfate precipitation, by ethylene glycol precipitation, or by a combination of these or with DEAE ion exchange chromatography, the IgG isolated does not retain the same physiologic characteristics it possessed while part of the fresh plasma. Upon intravenous administration, these preparations frequently result in fever, abdominal pain, vomiting, loss of consciousness, and circulatory collapse. Over 25% of the injected material is cleared from the circulation within minutes, with the remainder having a turnover time of less than three weeks. All of the above fractionation procedures, while isolating and concentrating the predominant neutralizing/protective antibodies as evidenced by in vitro neutralizing assays, impair the biologic effectiveness of the IgG molecule by virtue of altering the native physiologic state of IgG. The IgG isolated contains aggregated IgG, in quantities which indicate extensive denaturation. Up to 25% of IgG aggregates are contained in commercial preparations as well as by those prepared on a small scale. During the isolation procedure there is also evidence that plasminogen of plasma is activated to plasmin and has partially degraded the IgG molecule, leading to aggregation and structural alterations reflected by alterations in effector functions of the IgG molecule. These include altered anticomplementary activity, shortened turnover time or half life, and activation of the blood coagulation and kininogen systems in the body on intravenous injection resulting in a shock-like syndrome.

STRUCTURE AND FUNCTION OF THE IgG MOLECULE

The IgG molecule has a molecular weight of 160,000 Daltons. It is predominantly protein, but contains 3% carbohydrate. IgG differs from protein enzymes which have served as model proteins in that IgG molecules are biologically active proteins carrying out many different biologic functions. These biological functions include the primary function--combination of the IgG molecule with antigen, and secondary or effector functions such as immunoglobulin or IgG turnover, placental and gut active transport of the IgG molecule, activation of complement by the IgG molecule, cytophilic reactions involving the IgG molecule, metabolism and transport of the IgG molecule including functions which determine its half life in the circulation, and finally complement fixing mechanisms.

PRIMARY AND SECONDARY STRUCTURE

The IgG molecule can be regarded as derived from a basic structure of two light polypeptide chains and two heavy polypeptide chains linked together by covalent bridges of cystine residues. The four chains are paired so that the molecule consists of two identical halves, each of which has one heavy and one light chain. By amino acid sequence analysis, it is shown that both heavy and light chains have regions in the N-terminal portion with highly variable amino acid sequences and regions in the carboxy terminal end with constant amino acid sequences.

TERTIARY STRUCTURE

Considerable evidence from a variety of chemical and physical studies indicates that the heavy and light chains are folded into a linear series of compact globular regions called domains. The light chain is composed of two domains: one corresponding to the variable amino acid sequence region, the V_L, and the second is the constant amino acid sequence region, the C_L. In the heavy chains there are four domains, one formed by the variable region, V_H, and three by the constant homology region, $C_{\gamma 1}$, $C_{\gamma 2}$ and $C_{\gamma 3}$. Each three dimensional domain corresponds approximately to 110 amino acid residues, contains a single intrachain disulfide bond, and is linked to the neighboring domains by more loosely folded stretches of polypeptide.

CORRELATION OF STRUCTURE WITH BIOLOGIC FUNCTION

As a corollary to this structural model, it has been proposed that each domain evolved to perform specific functions, and thus the V_L and the V_H jointly form the antigen binding site where the constant region domains, particularly in the FC region, the $C_{\gamma 2}$ and $C_{\gamma 3}$, mediate the biologic effector functions of the immunoglobulin molecule. There is evidence which supports this concept of structural differentiation of the IgG molecule, and more specifically, that this modular structure is accompanied by functional differentiation within the IgG molcule. A number of investigations have shown that most of the interactions with the non-specific or

effector mechanisms of the body occur through the FC region in the molecule. In some cases the locations of these sites have been precisely defined. For example, it has been shown that the $C_{\gamma 3}$ domain is involved in the histamine release for mast cells in heterocytotrophic passive cutaneous anaphylaxis and in the binding to heterologous and homologous macrophages. It has also been determined that the site controlling catabolic rate, antibody dependent cytotoxicity, and complement fixation are likely to be associated with the $C_{\gamma 2}$ domain or to depend on the presence and the integrity of both domains since these activities are not displayed by isolated $C_{\gamma 3}$, or the PFC.

NATIVE, UNDENATURED MOLECULES REQUIRED FOR FULL NORMAL BIOLOGIC ACTIVITIES

The domain hypothesis proposes that the homology regions of the immunoglobulin molecule have evolved independently to perform separate functions. More recent work suggests that the roles played by the individual domains and the mediation of effector functions are in many cases more complex than those envisioned in the domain hypothesis. Although some functions such as C^{1Q} binding require only one domain, other activities are dependent upon higher orders of structure involving pairs of domains, or more likely the native conformation of the complete IgG molecule.

Finally, there is strong evidence to indicate that minor modifications in the domain structure resulting from methods of fractionation or from limited proteolysis by proteolytic enzymes alter the IgG molecule from its native, physiologic state in the blood and result in marked alteration in complement binding, anticomplementary activity, and turnover rate.

ISOLATION AND PURIFICATION OF THE NATIVE IgG MOLECULE

Methods to isolate IgG from plasma in its native, physiologic state must take into consideration conditions when denaturation occurs and must take into account specifically the area or areas of the IgG molecule most susceptible to alteration. The heavy chains of the IgG molecule contain a length of polypeptide chain, between the $C_{\gamma 1}$ and $C_{\gamma 2}$ domains, known as the hinge region. In this region there is flexibility between the antigen combining region (FAB) and the complement fixing segment (FC). The hinge region is rich in proline amino acid residues and contains the inter-heavy chain disulfide bridges. Under conditions of controlled denaturation, IgG can be enzymatically cleaved at points lying between domains in preference to those lying within domains. (This suggests that domains form a relatively compact structure joined by short sections of looser structure.) The hinge region appears to be the most susceptible part of the molecule to denaturation and enzymatic cleavage. Fragmentation with papain, pepsin, or plasmin has generally been used.

The IgG antibody molecule's primary function, namely its combination with antigen which occurs in the FAB portion, is most resistant to denaturation (or other conditions altering shape or causing change within the molecule). While the primary function remains unaffected by denaturation, the hinge region and constant domains of the FC region are markedly altered, an effect reflected by marked changes in such biologic effector functions as IgG turnover rate, metabolism, and complement fixation, and normal interactions with the complement pathway, the coagulation, fibrinolytic, and kininogen systems. In the past undue weight has been given to the fact that IgG molecules isolated by many different methods are still able to interact with antigen in an apparently unimpaired, native way. More attention must be given to the extensive evidence that other functions of the IgG molecule have been markedly impaired by alcohol fractionation, ammonium sulfate salting out method, polyethylene glycol method, etc., methods that all isolate IgG with little impairment in its ability to combine with antibody, but have marked denaturating effects on the other portions of the molecule. Denaturation as reflected by the presence of up to 25% aggregates, significantly decreases half life in the circulation, and significantly increases complement fixing activity.

STANDARDS FOR THE NATIVE ISOLATED IgG MOLECULE

The standards characterizing the native IgG molecule must include comparisons with IgG prepared by the current and prior art with IgG prepared by the method claimed in my aforesaid application Ser. No. 829,565 with final proof resting on the determinable characteristics and properties of the molecule in fresh plasma. While some modifications of IgG from its native state can be directly demonstrated by structural studies and in vitro tests of biological effector functions, the final definition of its native state must include purity and structural analysis, coupled with comparative studies between the different IgG's and IgG in fresh human plasma particularly when each is intravenously administered.

Purity—The current standards of purity whereby contaminants present in no lower than microgram concentrations are identified cannot be used since it has been demonstrated that the current IgG preparations contain nanogram quantities of peptides with potent pharmacologic activity. The administration of these materials produce severe vasoactive reactions. Therefore the purity standards for native, non-denatured monomeric IgG must be at lower levels to include an analysis of these substances.

Structural Analysis—Structural parameters defining altered or denatured IgG are at best rather ill defined, but include such gross characteristics as: (1) molecular weight characterization of the different molecules making up the IgG preparation including both high molecular weight aggregates and low molecular weight fragments, (2) anti-complementary activity, (3) native electrophoretic mobility ranges, (4) native isoelectric point ranges, and (5) demonstrating that the monomeric 160,000 molecular weight IgG molecules are resistant to limited proteolysis by plasmin.

In Vivo Characterization—The denatured non-native IgG molecule exhibits a number of altered biologic effector functions. Behavior of such IgG in the circulation of the body can only be assessed by comparative in vivo studies involving intravenous administration of both the IgG of fresh plasma and the purified IgG preparation. These include: (1) demonstration that the preparation is safe for intravenous administration in quantities of up to 200 mg/kg and does not produce either circulatory collapse, fever, pain, vomiting, or activate the kinin in the complement systems, and (2) half-life or turnover times similar to IgG of normal plasma.

While some modifications of the IgG molecule from the native state can be directly demonstrated by struc-

tural studies and in vitro testing, the final definition of the native molecule must include both structural and biologic evidence that on intravenous administration the IgG molecule behaves in a similar way to the IgG of fresh human plasma. The structural parameters denoting the native, undenatured, unaltered IgG molecule include: (1) native electrophoretic ranges, (2) native isoelectric ranges, (3) molecular weight of 160,000 Daltons with no aggrevated IgG and no IgG fragments, (4) low anti-complementary activity, and (5) resistance to limited proteolysis with plasmin. The biologic indicators denoting the native, undenatured, unaltered IgG molecule include: (1) normal half-life or turnover time following intravenous administration of three weeks, (2) no evidence of early accelerated (within minutes) phase of rapid clearance following intravenous administration (indicative of IgG partially degraded by plasmin and aggregated IgG), (3) safe for intravenous administration in large quantities (200 mg/kg) in the clinical setting (with clearly demonstrated freedom from untoward reactions such as fever, vomiting, loss of consciousness, etc.), and (4) free of properties activating the complement pathway, the blood coagulation and kininogen system.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further illustrated by the accompanying drawings in which:

FIGS. 1A through 1F represent the elution curves derived from chromatographic analysis of various IgG preparations compared to native IgG, FIG. 1A constituting the prototype for native human IgG;

FIG. 1B representative of elution curve of human IgG prepared by alcohol fractionation followed by reduction and alkylation;

FIG. 1C is a protein elution curve from a Biogel column of a fractionated human IgG that has been treated with β propiolactone;

FIG. 1D is a representative elution curve characteristic of IgG prepared by methods involving ethyl alcohol precipitation, ethylene glycol precipitation, and salting out procedures;

FIG. 1E is an elution curve from an ethyl alcohol fractionated IgG that has been degraded by plasmin activated during the fractionation procedure;

FIG. 1F is an elution curve of alcohol precipitated IgG analyzed in total protein to determine the distribution of various molecules, and concentration of the various peaks in terms of IgG, A, and M; and

FIGS. 2A through 2D show the differential effect of plasmin on denatured IgG and native IgG.

CHARACTERIZATION OF THE AGGREGATION, FRAGMENTATION AND SHAPE CHANGE OF THE VARIOUS IgG PREPARATIONS WITH COMPARISON TO NATIVE IgG

Exclusion chromatography using acrylamide copolymer beads (Biogel A 1.5) was selected as the method to determine the number of different molecular weight components in each IgG preparation and to determine if possible detectable changes in molecular shape. Since plasma contains numerous different proteins with molecular weight similar to the 160,000 Dalton IgG, the prototype for the native IgG was prepared from human plasma by the method incorporated in my aforesaid application Ser. No. 829,565. Comparisons under identical conditions of protein concentration, gel type, column diameter and length, etc. were made with a representative ethanol fractionated IgG, with a reduced and alkylated intravenous IgG, with chemically altered β propiolactone treated IV IgG and an ethanol precipitated plasmin degraded IgG. Quantitative assessment of the percent aggregation, percent fragments and alterations in molecular shape were determined by placing 2 ml samples of IgG at a concentration of 45 mg/ml over a Biogel A 1.5 column. The column was 2.6 cm in diameter by 94 cm in length. The emerging protein was collected in 7 ml aliquots and protein concentration determined by absorbance at 280 millimicrons or by quantitative determination of IgG content in Mancini plates.

With this method molecular weights plus molecular shape and hydration affect the behavior of molecules during chromatography. In general, protein molecules appear in the column eluate in order of decreasing size. The columns used had been standardized and calibrated for molecular weight determination using a number of different standardized protein molecules.

FIG. 1A represents the elution curve derived from this type of structural analyses and constitutes the prototype for native human IgG. The IgG was prepared by the method incorporated in application Ser. No. 829,565. The protein elution curve is symmetrical indicating monomeric molecular species of identical shape and volume. Aggregates constitute less than 1% of the total protein eluted. Fragmented IgG also constitutes less than 1% of the total protein added. The protein elution curve is consistent with a monomeric, unaggregated, unfragmented native IgG. This elution curve is to be contrasted with the elution curve for the following non-native IgG preparations.

FIG. 1B is representative of elution curve of human IgG prepared by alcohol fractionation followed by reduction and alkylation. The elution is not symmetrical. High molecular weight aggregates constitute 20% of the total protein of this preparation with a very high percent of high molecular weight (over 1,000,000) aggregates.

FIG. 1C is a protein elution curve from a Biogel column of a fractionated human IgG that has been treated with β propiolactone. This IgG contains over 14% aggregates, some consisting of very high molecular weight materials. It also contains detectable levels of fragmented 50,000 molecular weight IgG (2.5%). The main peak rather than being symmetrical is altered and consists of only 83% of the total protein.

FIG. 1D is a representative elution curve characteristic of IgG prepared by methods involving ethyl alcohol precipitation, ethylene glycol precipitation, and salting out procedures. Protein concentration, elution and column dimensions are identical on all runs. The protein elution curve is not symmetrical. Sixteen percent of the total protein is composed of various high molecular weight aggregates. These aggregates include dimers, trimers, tetramers, and molecular aggregates greater than 1,000,000 molecular weight. There are not appreciable quantities of fragmented IgG in this preparation. The elution is not symmetrical.

FIG. 1E is an elution curve from an ethyl alcohol fractionated IgG that has been degraded by plasmin activated during the fractionation procedure. While there is a reduced amount of aggregates, 27% of the total protein consists of IgG fragments with a molecular weight of around 52,000 Daltons. The ethyl alcohol precipitated plasmin degraded preparation shows both

aggregates and fragments. This method of treating alcohol fractionated IgG with plasmin has been attempted and materials of this type have been tested clinically to determine their suitability for intravenous administration. It is difficult to control the amount of proteolysis. It is also clear from published studies that plasmin degraded material is cleared very rapidly from the circulation so that large doses of material must be administered to achieve therapeutic levels.

FIG. 1F represents Biogel elution curve of an EtOH precipitated IgG analyzed in two ways-first, total protein to determine the distribution of various molecules, and second, concentration of the various peaks in terms of IgG, A and M. The IgG, A and M content of each fraction was determined by diffusion in Mancini plates and a curve constructed from data derived from this information. As it can be seen, the high molecular weight aggregates as well as the tetramers, dimers, trimers, are all composed of IgG and not contaminating proteins.

This type of structural analysis demonstrates the extreme susceptibility of the human IgG molecule to alteration by a number of currently practiced isolation procedures. Precipitation of IgG with ethyl alcohol, chemical modification with β propiolactone, and reduction and alkylation of the molecule all result in significant quantities of aggregates. (See Table I for comparison with native IgG.) In addition, there is evidence that these procedures result in marked shape changes as is reflected by the asymmetry of the elution curve, of the main 160,000 molecular weight components. Since both fragments and aggregates are eliminated from the circulation within minutes, there is also a drastic reduction in the quantity of 160,000 molecular weight molecules that would remain in the circulation and have some beneficial effect. The altered shape and the significant aggregation is strong supporting evidence that these fractionation procedures result in extensive denaturation of the IgG molecule.

THIN-LAYER POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING OF IgG: COMPARISON OF NATIVE IgG WITH CHEMICALLY ALTERED, STRUCTURALLY MODIFIED, AND DENATURED IgG

Proteins in solution have an electric charge. When these solutions are placed between two electrodes connected to a source of direct current, the proteins will migrate to the electrode of opposite charge. Thus, positively charged ions migrate to the cathode and are therefore termed cations, and negatively charged ions migrate to the anode and are termed anions. Generally proteins migrate to the cathode in acid solutions and toward the anode in alkaline solutions. There exists a pH value at which a given protein would not move toward either electrode. This pH or pI is defined as the isoelectric point and is a constant which can aid in the characterization of the protein. Isoelectric focusing is a very high resolution technique for the separation of proteins according to their isoelectric properties. Because of their heterogeneity, immunoglobulins and IgG in particular have broad isoelectric pH ranges rather than a narrow point such as would be the case for a homogeneous protein like serum albumin. However, even with the broad ranges, this method can be used to characterize most particularly the IgG's that have been chemically modified in preparation. When these chemical modifications alter the number of charged groups on the protein molecule, this is reflected by a new isoelectric point range. The method also is very sensitive in detecting alterations that contribute to the heterogeneity of immunoglobulins. The heterogeneity of pooled native IgG when focused results in a broad isoelectric spectrum of what appears to be homogeneous protein (with broad rather than sharp narrow bands). When the IgG has been modified, either chemically or broken down structurally to fragments, this introduces an additional type of heterogeneity—a microheterogeneity generated by alteration of the native IgG and is reflected by the introduction of a number of sharply defined narrow bands of various isoelectric points.

Analytical isoelectric focusing has been performed in polyacrylamide gel. The gel matrix stabilizes the pH gradient against convection. Polyacrylamide gel has most of the properties needed as a support for electrofocusing. It is desirable that the gel have a sufficiently large pore size to permit free passage of the proteins during focusing. The polyacrylamide gel for the electrofocusing to be described was cast in a 2 mm layer on glass plates.

During isoelectric focusing, proteins migrate electrophoretically in a stationary pH gradient generated by a number of low molecular weight ampholytes of varying IEP's. A steady state is reached in which sample proteins are concentrated or focused as bands at their isoelectric point. The gel used in this experiment (LKB 1804-101) contains a polyacrylamide concentration of 5%, an ampholyte concentration of 2.4% (w/v), is 3% crosslinked and is 235×90×1 mm in size. An LKB 2117 Multiphor unit is used in this experiment along with an LKB 2103 power supply. Filter paper tabs (5×10 mm—Whatman, Paratex) containing 20 μl of sample (0.1 mg protein) are used for sample application. The β propiolactone IgG was applied near the cathode while the remaining samples were applied near the anode. The samples were focused at 4° C. for 90 minutes at a constant 30 watts. The application tabs were removed after 45 minutes and the pH gradient of the gel at 4° C. determined at the end of the run. The pH is determined every 10 mm using a Beckman 3500 meter with a 5 mm combination electrode (Beckman 39505). The pH of the plate ranges from 4.9 to 8.9. The plate is refocused for 10 minutes and then the proteins are fixed by placing the gel in a solution containing 1% methanol (v/v) and 0.5% sulphosalicyclic acid (w/v) for 45 minutes. Prior to staining, the plate is soaked in destaining solution (25% ethanol [v/v] and 8% acidic acid [v/v]) for five minutes. This step allows the plate to equilibrate to staining conditions and allows for the complete removal of ampholytes from the gel. The plate is stained for 10 minutes at 60° C. in a filtered preparation containing 0.1% (w/v) Coomassie Blue R-250 dye in destaining solution. The plate is destained for at least 48 hours with several changes of destain.

The isoelectric range of various preparations of human IgG is presented in Table II. Note that the native IgG sample #5 has a pI range of pI 6.4–8.8. The β propiolactone treated IgG sample #1 with pI range of 5.6–7.2 demonstrates that there has been significant chemical modification of surface charge markedly altering the pI range from the native state. The reason for this is that β propiolactone reacts with carboxyl, amino, hydroxyl, sulphydryl, and phenolic groups of proteins. EtOH purification of IgG has a somewhat nearer range than the native material; however, the EtOH plasmin degraded sample #6 has the broadest range of 6.18–8.8.

The polyacrylamide focused gel from which these ranges were derived demonstrates another feature of this type of an analysis, namely the micro-heterogeneity of each preparation. There are two procedures which introduce significant micro-heterogeneity to the IgG-β propiolactone treatment sample #1 and plasmin degradation sample #6. The sharp dense bands in the plasmin treated sample are derived from fragmentation of the aggregates and degradation of some of the 160,000 molecular weight molecules and contribute to the heterogeneity seen here. These fragments would of course be cleared very rapidly from the circulation and would not be there for therapeutic effect. The β propiolactone treated IgG demonstrates the most marked alterations of the molecule both in IEP and in micro-heterogeneity. What effects these would have on introduction in the host is not clear. However, one would expect an impairment of some of the effector functions of the molecule and significantly reduced half-life in the circulation. In addition, alteration of surface charges by β propiolactone indicate the native groups have been replaced by foreign chemical groupings, charges modified, and could post added hazards by introduction of foreign antigenic determinants that could well generate antibody which would react with the β propiolactone treated molecule.

TABLE I

MOLECULAR SPECIES IN DIFFERENT HUMAN IgG PREPARATIONS: COMPARISON OF AGGREGATES, MONOMERS, AND FRAGMENTS WITH NATIVE IgG

Preparation	Aggregates >300,00 MW %	Monomers 160,000 MW %	Fragments 50,000 MW %
Native human IgG IV	<1	99	<1
Reduced & Alkylated IgG IV	20	80	<1
β propiolactone IgG, IV	14	83	2.5
EtOH IgG, IM	16	84	<1
EtOH plasmin IgG, IM	7	63	27

2 ml sample applied to 2.6 × 94 cm Biogel A 1.5 column Protein concentration 45 mg/ml, Total Protein applied 90 mg

TABLE II

ISOELECTRIC RANGE OF VARIOUS PREPARATIONS OF HUMAN IgG

Sample #	Preparation	pI Range
1	β propiolactone IgG, IV preparation	5.6–7.2
2	Reduced & Alkylated IgG, IV preparation	6.5–9.0
3	EtOH IgG, IM preparation$_1$	6.7–8.7
4	EtOH IgG, IM preparation$_2$	7.0–8.8
5	Native human IgG, IV preparation	6.4–8.8
6	EtOH plasmin IgG, IM preparation	6.18–9.43

Sample protein concentration 5.0 mg/ml Total volume 20 µl, 0.1 mg total protein

IMMUNOELECTROPHORETIC ANALYSIS

Immunoelectrophoresis can demonstrate a number of factors, but for this study its purpose was to demonstrate two features—first, if there were any changes in the electrophoretic mobility of the gamma globulin suggesting chemical modification, and second, if there were any impurities present in the gamma globulin. Although this method is not the most sensitive for detecting impurities, it is possible to pick up contaminants that are present in microgram quantities.

In immunoelectrophoresis two methods are combined, gel electrophoresis followed by immunodiffusion. A buffered, agarose gel is used as the medium and the two steps of analysis are performed sequentially. Buffered agar is poured onto a horizontal glass plate to a uniform thickness. Following solidification and curing, a longitudinal center trough is cut out in the midline and a well punched out on either side of the trough for electrophoresis of the serum sample. After the serum is placed in the center well, it is separated under the influence of an electric field, and the charged protein particles migrate. Among the factors governing the migration may be the charge, size and shape of the particles, the concentration, ionic strength, pH of the solvents, the temperature and the viscosity of the medium, and the character and intensity of the electrical field. Following 80 minutes at 150 volts at which time it has been found that the proteins under analysis have all reached desirable separation, the slide is taken out of the electric field and an antisera specific to either human gamma globulin or whole human serum protein is added to the center trough and allowed to diffuse into the agar and react with each one of the separated plasma protein constituents. Following a 24 hour period for equilibrium and diffusion of the antisera into the gels, the gels are then washed with saline for a 48 hour period to remove any unreacted protein, then stained with an amido black dye, dried and photographed.

ELECTROPHORETIC MOBILITY

Electrophoretic mobility and impurities contaminating various IgG preparations can be visually compared with native IgG in the human plasma. Immunoelectrophoresis of the samples against an antisera was observed which reacts only with human IgG. The electrophoretic mobility of native IgG sample #4 and plasma IgG samples #6 and #7 were compared with the other IgG preparations. Samples were all at a protein concentration of 10 mg/ml. Sample #1 contained the β propiolactone treated IgG and had clearly altered electrophoretic mobility whereas the reduced and alkylated sample #2, the ethyl alcohol samples #3 and #5 showed relatively similar electrophoretic mobility to the native IgG and the IgG in plasma. The alteration of the electrophoretic mobility of the β propiolactone treated IgG would be expected from the demonstrated changes in IEP.

The precipitation bands from the immunodiffusion part of the immunoelectrophoresis performed with anti-human IgG showed only the presence of the IgG in each preparation including whole human plasma. To demonstrate the presence of other proteins or impurities in each sample the immunodiffusion part of the immunoelectrophoresis must be performed with anti-human whole serum. The anti-whole serum antibody is added to the trough because it contains antibodies to at least 30 different human plasma proteins and can therefore be used to determine impurities in each sample. The sample protein concentration in one experiment was 10 mg/ml in order to demonstrate the complexity of proteins in plasma while the sample protein concentration in a parallel companion experiment was 40 mg/ml to demonstrate the extent of impurities in each of the IgG preparations. It was shown that in addition to the introduction of additional negative charges, that the β propiolactone treated IgG sample #1 contains a significant contamination with another anodally migrating protein. Sample #2, the reduced and alkylated IgG, shows splitting of the main IgG band indicating partial fragmentation.

Where the protein concentration of the sample had been increased to 40 mg/ml, we see better the extent of contaminating proteins in the various IgG preparations. The β propiolactone treated IgG sample # 1 contains in addition to the two major components at least two other protein ares adjacent to the well. Sample #2, the reduced and alkylated IgG, also is contaminated with these anodally migrating proteins including a band near the well. Sample #3, ethyl alcohol precipitated human IgG preparation, contains small quantities of the anodally migrating component plus some splitting of the main band and two additional minor bands near the well. The native IgG (sample #4) can be seen as homogeneous and consists of IgG. The fifth well contains another ethyl alcohol treated ethyl alcohol human IgG indicating less contamination than in the other preparations. It is to be noted that there is some variation from manufacturer to manufacturer in these proteins and that some practice a higher degree and more sophisticated art than others.

The immunoelectrophoretic analysis of the different IgG preparations confirms the fact that β propiolactone isoelectric point alterations are reflected in changes in electrophoretic mobility. In addition it demonstrates that the reduced and alkylated as well as some of the alcohol fractionated material contain a splitting of the IgG main band and more importantly that with the exception of the native IgG, these other preparations contain varying degrees of contaminants, from those present in milligram quantities to those detectable in microgram quantities. It is to be anticipated that because of the relative impurity that there are a number of other undetectable contaminants present in nanogram levels in these preparations.

QUALITATIVE ASSESSMENT OF THE EXTENT OF THE DENATURATION OF IgG: COMPARISON OF EtOH PREPARED IgG WITH NATIVE IgG-DETERMINATION OF DENATURATION BY SUSCEPTIBILITY TO LIMITED PROTEOLYSIS BY PLASMIN

Proteins, particularly those that exhibit characteristic biologic activity as enzymes, immunoglobulins, and IgG in particular, usually lose some if not all activity on denaturation. Denaturation may be caused in various ways. Among these are heating, treating with acid, alkali, or organic solvents such as ethyl alcohol, and finally concentrated solutions of salt or dilute concentrations of protein in solution. All these treatments will cause an alteration in the solubility properties of most proteins, but proteins show a wide difference in sensitivity to any one of these methods of denaturation. In practice if treatment is not unduly prolonged, the denaturation may be reversed by restoring the condition at which the protein is stable; therefore, we can distinguish between reversible and irreversible denaturation of proteins. In theory all denaturation is reversible.

The change in shape and the presence of aggregates on Biogel elution heretofore described for different IgG's, suggested that the IgG's were altered and denatured by methods of isolation and purification. A more precise and quantitative method of determining the extent of denaturation of IgG was therefore explored. Since the essential feature of the denaturation process of proteins is associated with an unfolding of tightly coiled peptide chains leading to the disorganization of the internal structure of the protein, we look for other means of determining denaturation other than decreased solubility, change of shape and greater asymmetry. It is well known that denatured proteins generally are more susceptible to the attack of proteolytic enzymes and native proteins. The unfolding of the native protein makes the peptide bond more accessible to enzymic action; therefore, one would expect denatured proteins to be more susceptible to the attack of proteolytic enzymes than the native proteins.

The method developed to quantitate the extent of denaturation of various IgG preparations for comparison with native IgG utilizes limited proteolysis with the enzyme plasmin. Plasmin is an endopeptidase and cleaves arginine-lysine or valine-isoleucine peptide bonds. The limited proteolysis test with plasmin was achieved by incubating at 37° C. a solution of each IgG preparation in a 0.05 M Tris HCl, 0.2 M NaCl, 0.02 M glycine buffer (pH 8.0) 0.5 ml with human plasmin enzyme solution 300 units containing 8.3 mg/ml of activated plasmin. The plasmin was activated by incubating with 2330 units of urokinase. The reaction of IgG and plasmin contained 105 mg IgG/2.5 mg plasmin, a ratio of 42. The reaction was stopped at 18 hours by freezing the samples at −70° C. and then at a time when they could all be run together, they were eluted from a Biogel A 1.5 2.6×94 cm calibrated column. The effluent was collected in 7 mm aliquots and the optical density determined by adsorption at 280 millimicrons. The percent of protein in each of the peaks was calculated by determining the total area of the eluted peaks by planimetry and approximating areas of each peak's overlap.

The elution of protein from the Biogel A 1.5 column is illustrated in FIG. 2. Native IgG (FIG. 2D) incubated at 37° C. for 18 hours at a concentration of 45 mg/ml without plasmin elutes from the Biogel in a symmetrical, uniform peak of molecular weight of 160,000 Daltons, no aggregates nor fragments. Following limited proteolysis with plasmin (FIG. 2B), the native IgG appears unaltered, the peak is still symmetrical with little or no evidence of fragments or aggregates. This demonstrates that under conditions of this experiment the IgG preparation was not acted on by plasmin and therefore is a native IgG.

The elution of the EtOH IM IgG that was incubated for 18 hours at 37° C. with no enzyme is illustrated in FIG. 2C. There is extensive aggregation including dimers, trimers, and high molecular aggregates. The peak shows distinct asymmetry. Incubation of this IgG preparation with plasmin under the identical conditions as the native IgG is illustrated in FIG. 2A. Results of this study show the following: (1) disappearance of most of the aggregates, (2) the reduction in the main 160,000 molecular weight peak, and (3) formation of a second peak containing 52,000 molecular weight fragments of IgG. Quantitation of the differential effect of plasmin on EtOH IM IgG and native IgG is presented in Table III. Native IgG is resistant to limited proteolysis by plasmin whereas plasmin attacks and degrades the EtOH IM IgG. Aggregates which made up 13% of this preparation are attacked and end up as 52,000 molecular weight fragments. More significantly, the main 160,000 molecular weight peak is sensitive to plasmin proteolysis to the extent that 39% of this material is hydrolyzed and appears as the 52,000 molecular weight fragments. This is particularly informative since it confirms that the asymmetry of this peak that suggested denaturation clearly represents extensive unfolding of the polypeptide chains to the extent that plasmin now can attack and degrade this 160,000 molecular weight component

since approximately 40% of the 160,000 molecules are now susceptible to proteolysis by plasmin. These are representative results of a number of experiments confirming that other methods of preparing IgG β propiolactone, reduction and alkylation, salting out, ethylene glycol precipitation denature the protein and render them susceptible to the attack by the proteolytic enzyme plasmin while the native IgG is unaffected.

TABLE III

DIFFERENTIAL EFFECT OF PLASMIN ON EtOH IM IgG AND NATIVE IgG

Sample	Aggregates	160,000 Peak	52,000 Peak
EtOH IgG IM	13%	87%	less 1%
EtOH IgG IM + Plasmin	1%	48%	51%
Percent 160,000 peak attached by plasmin		39%	
Native IgG	less 1%	99%	0%
Native IgG + Plasmin	0%	98%	1%
Percent 160,000 Peak attached by plasmin		1%	

IN VITRO ASSESSMENT OF ALTERED BIOLOGIC EFFECTOR FUNCTIONS OF VARIOUS IgG PREPARATIONS COMPARED WITH NATIVE IgG: ANTI-COMPLEMENTARY ACTIVITY AS AN INDICATION OF DENATURATION

In its normal state in blood, IgG does not interact appreciably with components of the complement system. The biologically active sites within the FC region ($C_{\gamma 2}$ domain) of IgG that interact with complement are expressed or exposed as a result of conformational or structural changes that normally result following specific antigen/antibody combination with surface antigens on bacteria, viruses, altered cells. Results of this combination normally mediate many aspects of inflammation and facilitate ingestion of pathogens by phagocytes. Once the site in the FC region ($C_{\gamma 2}$ domain) is exposed, the first component of complement C1 attaches through a non-covalent linkage or ionic bond and results in enzymatic activation of the complement cascade. Structural alterations of the IgG resulting from denaturation by solvent precipitation such as aggregate formation also expose these biologically active complement fixing sites. Therefore the extent of complement fixation or anti-complementary activity of IgG can be used as a measurement of denaturation and alterations from the native state.

It was observed in 1944 that fractionated IgG was anticomplementary. Subsequently in 1945, it was demonstrated that aggregates in the fractionated IgG were responsible for this effect. Later in 1959 it was shown that heat denatured (aggregated) IgG fixed complement in a manner indistinguishable from that of antigen/antibody complexes.

The methods used in these studies to compare the anticomplementary activity of various IgG preparations with native IgG involved the determination of the quantity in micrograms of IgG protein required to fix 50% of a standard preparation of C1 complement. Comparisons were made of anti-complementary activity of human IgG preparations with native IgG by determining the micrograms of IgG protein required to fix 50% of C1. Table IV shows results of these determinations. With the exception of the EtOH plasmin IgG IM preparation, there is a direct relationship between the aggregate concentration and the anti-complementary activity. This is particularly noteworthy since plasmin degradation removes aggregates and reduces anti-complementary activity. This method has been used in the preparation of intravenous IgG. However, there have been many problems associated with this because the extent of degradation of the main 160,000 molecular weight IgG cannot be adequately controlled. This results from the fact that the main peak is also extensively denatured as heretofore shown. The EtOH Red Cross IgG containing 30% aggregates was the most active in fixation of C1 complement since only 0.15 microgram fixed 50% of the complement. Also of interest is the fact that while there is a direct general relationship between the extent of aggregation and the anti-complementary activity, the quantity of the EtOH reduced and alkylated IgG IV for fixation of complement required twenty times the quantity of protein for 50% complement consumption. The purpose of reduction and alkylation of this IgG was to reduce its anti-complementary activity and this is demonstrated and supported by these studies. However, the native IgG has significantly less anti-complementary activity (400-fold less) than any of the other preparations. These analyses add further support to the fact that a great number of fractionation procedures produce denaturation in the IgG molecule, particularly when compared to a native IgG. These observations are consistent with the view that there have been extensive alterations in the structure of these molecules, particularly in the FC region that result in exposing the biologically active complement fixing sites and provide information explaining why in intravenous administration these preparations of altered, denatured IgG produce significant systemic reactions, one of which involves the activation of the complement cascade. It should be noted that plasmin degradation, reduction and alkylation, and β propiolactone treatment of IgG reduce its anti-complementary while there are still appreciable quantities of aggregated IgG. Complement fixation therefore has some limitations in assessing denatured IgG.

TABLE IV

COMPARISON OF ANTI-COMPLEMENTARY ACTIVITY (C^1 BINDING) OF HUMAN IgG PREPARATIONS WITH NATIVE IgG

Preparations	Molecular Size	Percent Aggregation	Anti-Complimentary Activity*
EtOH IgG Red Cross	160,000 (69%)	30%	0.15
EtOH IgG IM	160,000 (84%)	16%	0.44
EtOH-Reduced & Alkylated IgG IV	160,000 (80%)	20%	8.00
EtOH-plasmin IgG IM	160,000 (63%) 52,000 (27%)	7%	22.00
Native IgG IV	160,000 (99%)	less 1%	500.00

*μg Protein required to fix 50% C_1^1q.

PHARMACOLOGICALLY ACTIVE IMPURITIES IN HUMAN IgG PREPARATIONS: COMPARISON OF NATIVE IgG WITH HUMAN IgG PREPARED BY CONVENTIONAL METHODS

Components of the fibrinolytic, coagulation and kininogen systems of plasma are activated during fractionation by ethyl alcohol precipitation, ethylene glycol precipitation, salt precipitation, and by DEAE chromatography. Because these activated components are not removed during fractionation, they appear in nanogram quantities in the final IgG product. However, due to their potent activity, they nonetheless have been implicated in the production of flushing, erythema, and hypotension leading to circulatory collapse when the IgG is injected intravenously. The native IgG product prepared by the method described in my aforesaid application Serial No. 829,565 does not produce any of these above reactions on intravenous administration and therefore is presumed to be free of these contaminants. There are two demonstrable reasons why this is the case: (1) the precursors of the fibrinolytic, coagulation and kininogen systems are removed by the silica dioxide (SiO_2) treatment of plasma before fractionation on QAE, and (2) the elution pH of the QAE ion exchange procedure retains proteins and peptides and the IEP range of these activated fragments (pI of 4.2–4.6). Table V shows results of treating plasma with 40 grams/liter of silica dioxide The pretreated plasma contains normal levels of the various precursors including the following: fibrinogen, plasminogen, factor XII (Hagamen factor), prekallikrein system, and the complement components. The post-silica dioxide treatment plasma is without detectable levels of these factors.

It has been proposed that the flushing, erythema, and hypotension result from the presence of Hagamen factor fragments (prekallikrein activator PKA) which by acting enzymatically on prekallikrein, continue to generate bradykinin in the patient in excess of that being inactivated. The following scheme illustrates part of the proposed mechanism:

MECHANISM FOR INDUCTION OF HYPOTENSION BY PKA ACTIVATOR IN IgG PREPARATIONS

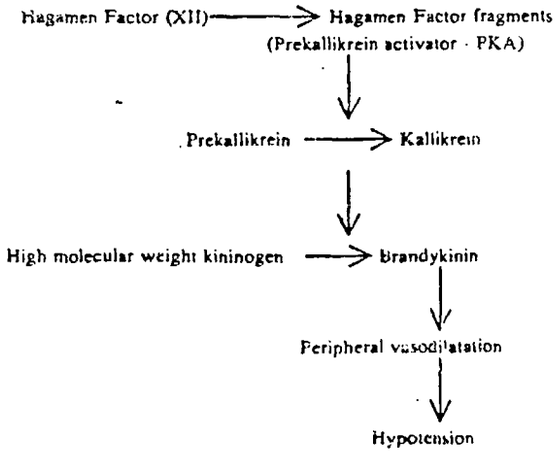


TABLE V

REMOVAL OF THE COMPLEMENT, COAGULATION AND KININOGEN COMPONENTS OF PLASMA WITH SiO_2

	Plasma	Post SiO_2 treated plasma
Total protein	64 mg/ml	70 mg/ml
Fibrinogen	300 mg %	0
Plasminogen	12 mg %	0
β-lipoprotiens	420 mg %	0
HDL associated HLA antigens	Present	Not detected
Cholesterol	160 mg %	0
Triglycerides	100 mg %	50 mg %
Clotting Factors	Percent Normal Activity	
II	100%	100%
V	100%	0%
VII	100%	0%
VIII	100%	0%
XI	100%	0%
XII (Hageman Factor)	100%	0%
Hemolytic C (screen)	Normal	No lysis
Hemolytic C (quant.)	24 units/ml	Not detected
C_4	480 μg/ml	<10
C_3	1200 μg/ml	<30
Clq	194 μg/ml	Not detected
C_5	112 μg/ml	"
Properdin	13.6 μg/ml	"
C_3PA	187 μg/ml	"
Prekalikrein	84* μmole/hr/ml	0*

*Kaolin activated

Table VI shows results of analysis for prekallikrein activity of native IgG and other IgG preparations. The native IgG was prepared by the method of my earlier application. It can be seen that the ethyl alcohol IgG preparation and the reduced and alkylated intravenous IgG both contain significant quantities of prekallikrein activator activity, while the native IgG and the β propiolactone preparations are essentially free of this activity.

INTRAVENOUS INJECTIONS OF NATIVE IgG AND OTHER ALTERED IgG PREPARATIONS: INCIDENCE OF SEVERE REACTIONS AND CIRCULATORY HALF-LIFE

Intravenous injection of fresh human plasma is safe and not associated with adverse reactions. The IgG present in fresh plasma has a circulatory half-life following intravenous injection of 24–32 days. Therefore, a native IgG preparation should be expected to possess similar characteristics upon intravenous administration. All priorities have been concentrated first on IgG preparations safe for intravenous administration with secondary emphasis on circulatory half-life. However, a native IgG preparation which combines safety and half-life comparable to IgG in fresh plasma would provide maximal benefits to the patients, namely safety and maximal therapeutic efficacy. The preceding sections have been devoted to studies determined to elucidate the many effects of various fractionation procedure in introducing structural alterations in the IgG isolated. Whether these documented, irreversibie alterations from the native state result in decreased circulatory half-life and the production of severe adverse reactions will be addressed in this final section. Table VII compares the circulatory half-lifes following intravenous administration of various IgG preparations. IgG contained in fresh normal plasma has a half-life of 24–32 days, while the half-life of native IgG is from 24-27 days. All other preparations have significantly reduced half-lifes. Plasmin degraded IgG fragments' half-lifes

range from 5 hours for the FAB fragments, 8–10 days for the FC fragment, and 18–20 days for the intact 160,000 molecular weight component. Alterations of surface groups, surface charge, isoelectric point, electrophoretic mobility, micro-heterogeneity, and aggregation by β propiolactone treatment of IgG result in circulatory half-life of 14 days. These reductions in circulatory half-life significantly reduce therapeutic activity of IgG. For example, the reduction from 28 days to 14 days in half-life would reduce the therapeutic index by a factor somewhat greater than 2. This would require twice the dose of IgG to achieve comparable therapeutic effects of a native IgG preparation.

TABLE VI

PREKALLIKREIN (PKA) ACTIVITY OF NATIVE IgG AND OTHER IgG PREPARATIONS

Preparation	PKA activity (% of reference)
Native IgG	<1
EtOH IgG IM	62
Reduced & Alkylated IgG IV	90
β propiolactone IgG IV	<1
Reference	100

TABLE VII

CIRCULATORY HALF-LIFE OF VARIOUS HUMAN IgG PREPARATIONS FOLLOWING INTRAVENOUS ADMINISTRATION

Preparation	Half-life (T1/2)
Fresh plasma (IgG)	24–32 days
EtOH plasmin IgG	
Intact IgG	8–20 days
FC fragment	8–10 days
FAB fragment	5 hours
β propiolactone IgG	4–12 days
Native IgG	23–27 days

INCIDENCE OF ADVERSE REACTIONS

The high incidence of severe adverse reactions on intravenous administration of the licensed ethyl alcohol IgG has prevented administration by this route. The incidence of these reactions is presented in Table VIII. Depending on the patient population, reactions can vary from 92% in immune deficiency syndrome patients to 13% in normal healthy individuals, whereas normal plasma is not productive of reactions in either group. β propiolactone treatment of IgG reduces significantly the incidence of severe reactions in immune deficiency patients (from 92% to 15%). Intravenous administration of native IgG however approaches fresh plasma in almost complete absence of reactions. We conclude that the added efforts required to prepare an undenatured, monomeric, native IgG results in definite benefits to the patient. These include safety, significantly greater therapeutic index by virtue of the near normal circulating half-lifes.

TABLE VIII

INCIDENCE OF SEVERE REACTIONS ON INTRAVENOUS ADMINISTRATION OF VARIOUS IgG PREPARATIONS

Preparations	# Patients	% Reactions
Normal Fresh Plasma		
Immune deficiency syndrome patient	26	none
Native IgG		
Patients with life threatening infection	10*	<1%
β propiolactone IgG		
Immune deficiency syndrome patient	12	15%
EtOH IgG		
Normal, healthy patient	55	13%
Immune deficiency syndrome patient	15	92%

It is apparent that many modifications and variations of this invention as hereinbefore set forth may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only and the invention is limited only by the terms of the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An isolated natural, unaltered, unfragmented, undenatured monomeric immune gamma globulin for intravenous administration characterized by 99% purity and containing less than 1% aggregates and less than 1% dissociated materials.

2. Purified gamma globulin according to claim 1 further characterized by molecular weight of 160,000 Daltons with no high molecular weight aggregated IgG and no low molecular weight IgG fragments.

3. Purified gamma globulin according to claim 1 further characterized by native electrophoretic ranges.

4. Purified gamma globulin according to claim 1 further characterized by native isoelectric point ranges between about 6.4 and 8.8.

5. Purified gamma globulin according to claim 1 further characterized by resistance to proteolysis with plasmin.

6. Purified gamma globulin according to claim 1 further characterized by low anti-complementary activity.

7. Purified gamma globulin according to claim 1 further characterized by normal circulatory half-life of at least about three weeks following intravenous administration.

8. Purified gamma globulin according to claim 1 further characterized by freedom from properties activating the complement pathway, blood coagulation and kininogen system.

9. An isolated natural, unaltered, unfragmented, undenatured monomeric immune gamma globulin for intravenous administration characterized by 99% purity and containing less than 1% aggregates and less than 1% dissociated materials, having molecular weight of 160,000 Daltons with no high molecular weight aggregated IgG and no low molecular weight IgG fragments, and further characterized by native electrophoretic ranges, native isoelectric point ranges between about 6.4 and 8.8, resistance to limited proteolysis with plasmin, low anti-complementary activity, normal circulatory half-life of at least about three weeks following intravenous administration, and freedom from properties activating the complement pathway, blood coagulation and kininogen system.

* * * * *

IMMUNOGLOBULINS: CHARACTERISTICS AND USES OF INTRAVENOUS PREPARATIONS

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Preparation and Intravenous Use of Undenatured Human IgG[1]

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ABSTRACT

A stable immunoglobulin for intravenous use was prepared by chromatography of silicon dioxide-treated human plasma on QAE-Sephadex. This preparation has been used clinically in intravenous doses of 20–200 mg/kg for more than four years; it has not caused any severe reactions, nor has it transmitted viral hepatitis. Comparative studies showed this preparation to resemble the native IgG of fresh plasma more closely than any other intravenous IgG preparation tested. The IgG in this preparation shows the same distribution of electrophorectic mobilities as that in whole plasma, is low in anti-complementary activity, and is primarily in monomeric form. It is resistant to limited proteolysis, free of contaminating plasmin and preallikrein activator activity, and has a half-life of 18–24 days *in vivo*. The only aspect in which this preparation appears to differ from the IgG of unfractionated plasma is the absence of subclass IgG3. It otherwise contains all the major neutralizing antibodies normally found in immune globulins.

INTRODUCTION

Separation of IgG from the hundreds of different proteins in plasma in a manner that will yield an immunoglobulin product suitable for intravenous administration has presented major challenges to protein fractionation. These include isolation of IgG that is not denatured and is free of fragments or activated peptides that may develop during the purification process.

At present, the only licensed procedure for the fractionation of human plasma in the U.S. is the Cohn-Oncley cold alcohol method. Use of this process to prepare albumin for intravenous administration has been extremely successful. However, immune globulins isolated by this procedure must be administered intramuscularly. Intravenous administration can result in a series of undesirable side effects which include erythema, vomiting, abdominal pain, fever, hypotension and loss of consciousness. In marked contrast native IgG, in the form of fresh human plasma, can be administered intravenously with few untoward reactions.

The importance of these findings stems from the fact that there are a number of clinical situations, notably those occurring in immunocompromised patients, in which no adequate therapy exists for the treatment of certain life-threatening infections. The administration of large quantities of immunoglobulin intravenously may be efficacious in these situations (1). Such quantities cannot be given by infusing plasma without incurring the danger of circulatory volume overload.

There are also clear indications that the efficacy of intramuscular (IM) immune globulin for the prophylaxis of viral infections, both in immunodeficient patients and in normal individuals, is related to the quantities of passive antibody that enter and persist in the circulation. The IM route restricts the quantity of immune globulin that can be administered, prevents significant quantities from reaching the circulation, and retards the development of maximum blood levels for several days (3–6).

Two major factors determining the incidence and severity of adverse reactions to intravenous (IV) immune globulins are the patient population (2) and the fractionated preparation itself (2,4,5). IV administration of IM preparations to normal healthy volunteers produces severe reactions in 13%, whereas the incidence of severe reactions increases to 93% when identical preparations are given IV to immunodeficient patients (2). Undesirable consequences of plasma fractionation which may play some role in these reactions include the denaturation of significant quantities of immunoglobulin (7–10), the generation of pharmacologically active molecules from labile plasma proteins (11), and the inability to identify and remove these active contaminants from the final product.

[1] Supported in part by USPHS Grant AM 13083.

It appeared, therefore, that a method should be sought to produce an immunoglobulin preparation with properties the same as, or very similar to, those of the IgG in fresh plasma. The immediate goal was to prepare a stable, undenatured IgG that could be used for the treatment of life-threatening viral infections in chronically immunosuppressed renal transplant recipients. Infection is the most serious complication of immunosuppression and is the most common cause of death (12), and cytomegalovirus (CMV) infections have been established as a major cause of morbidity and mortality in these transplant patients (13). A number of potentially useful antiviral agents, such as idoxuridine (alone or in combination with novobiocin), cytosine arabinoside (ara-C), and adenine arabinoside (ara-A), have all been without effect (14,15). Since the fatal CMV syndrome ultimately results in hypogammaglobulinemia in 100% of the patients in the third week following diagnosis and is associated with complete loss of antibody to CMV and death in 100% of the patients in the fourth week (16), there was an urgent need for a preparation of human IgG which would be safe for intravenous administration in large doses (200 mg/kg) (1).

At the outset, criteria which had to be met before an intravenous human immune globulin preparation could be developed and used clinically were established. The criteria were (a) that the isolation method be adaptable to large scale production involving ≥100-liter pools of human plasma; (b) that labile plasma proteins, particularly those susceptible to activation, be removed before final fractionation; (c) that the fractionated IgG be HBsAg negative; (d) that the purification methods yield an unaltered, undenatured, pure IgG; (e) that the normal structural integrity of the Fc domains, which control the biologic effector functions of complement fixation and circulatory half-life, be retained; (f) that the preparation be low in anticomplementary activity; (g) that the preparation contain the predominant IgG subclasses in which the neutralizing antibodies of plasma reside; and (h) that the IgG be stable on storage and show no evidence of aggregate formation or fragmentation.

This paper describes a method for isolating native IgG from pooled human plasma. Results of structural and functional analyses of this IgG, of immune globulin prepared by ethanol fractionation, and of IgG modified by reduction and alkylation, or by plasmin or β-propiolactone treatment are also presented.

METHODS

Exclusion Chromatography

For assessment of aggregation and fragmentation, the IgG preparations (2 ml, 45 mg IgG/ml) were applied to a Biogel A-1.5m column (2.6 x 94 cm) that had been calibrated with proteins of known molecular weight. Protein concentration of the eluate fractions (7 ml each) was monitored at 280 nm; IgG concentration was determined by radial immunodiffusion in Mancini plates.

Thin-Layer Polyacrylamide Gel Isolelectric Focusing

The gel employed was prepared with 5% acrylamide, 0.15% N,N'-methylenebisacrylamide, and ampholytes (pH range, 3.5–9.5) at a concentration of 2.4% (w/v). Its dimensions were 235 x 90 x 1 mm. An LKB 2117 Multiphor unit was used with an LKB 2103 power supply. Filter paper tabs (5 x 10 mm—Paratex, Whatman) containing 20 μl of sample (0.1 mg protein) were used for sample application. The β-propiolactone IgG was applied near the cathode; all other samples were applied near the anode. The samples were focused at 4 °C for 90 min at a constant power of 30 watts. The application tabs were removed after 45 min, and the pH gradient in the gel at 4 °C was determined at the end of the run. The pH was measured every 10 mm by means of a Beckman 3500 meter with a 5 mm combination electrode (Beckman 39505); the gradient determined in this manner spanned the range from pH 4.9 to pH 8.9. After pH was measured, the plate was refocused for 10 min, whereupon the proteins were fixed for 45 min in a solution containing 1% (v/v) methanol and 0.5% (w/v) sulphosalicylic acid. Before staining, the gel was soaked in a solution of 25% (v/v) ethanol and 8% (v/v) acetic acid for 5 min. This step allowed equilibration to staining conditions and effected the complete removal of ampholytes. The plate was then stained for 10 min at 60 °C in a filtered solution of 0.1% (w/v) Coomassie Blue R-250 in the ethanol-acetic acid mixture described above. Afterward, it was destained for at least 48 h in this same mixture.

Limited Proteolysis Assay

A limited proteolysis test with plasmin was used to estimate the extent of IgG denaturation. It involved incubating a sterile solution of IgG at 37 °C in 0.05 M Tris-HCl, 0.2 M NaCl, 0.2 M glycine (pH 8.0) with human plasmin that had been activated with urokinase. A final 1-ml solution contained 30 mg IgG, 0.71 mg plasmin, and urokinase

(167 Plough units). After 18 h the reaction was stopped by freezing the samples at −70 °C. Fragmentation was assessed by exclusion chromatography on Biogel A-1.5m (see above). The distribution of protein among the peaks in the elution pattern was determined by planimetry after estimating the areas of overlap.

Anti-Complementary Activity

As an additional indication of IgG denaturation, the anti-complementary activity of various human immune globulin preparations compared with that of the IgG isolated in the present study. Complement studies were performed by Dr. Richard J. Pickering, Research Physician, New York State Department of Health, Albany, New York.

Prekallikrein Activator (PKA) Activity

Assays were performed by Dr. Barbara M. Alving, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland.

Antibody Activity

Assays of plasma pools and final IgG preparations were performed by Dr. Hilaire Meuwissen of the New York State Department of Health Laboratories, Albany, New York.

Other Analyses

Plasma proteins were quantitated with Behring radial immunodiffusion plates, unless otherwise noted. Cholesterol and triacylglycerols ("triglycerides") were determined by automated lipid analysis. Total protein was determined by the biuret method, and clotting factors were determined by means of assays that made use of specific factor-deficient plasma. The concentrations of complement components, prekallikrein, and properdin were determined by Dr. Henry Gewurz, Rush Presbyterian Medical Center, Chicago, Illinois.

EXPERIMENTS AND RESULTS

Preparative Procedures

Plasma Collection

Blood was collected from healthy volunteer adult donors by the St. Paul Red Cross, the University of Minnesota Blood Bank, and the Minneapolis War Memorial Blood Bank. The plasma, as obtained, was either outdated or cryoprecipitate-poor. It was frozen and stored at −20 to −70 °C. Only plasma tested and found negative for HBsAg was used.

Plasma Pooling

The contents of ~450 bags were thawed, pooled into a sterile container (plasma pool volume, 130 liters), and mixed. Samples were removed and tested for HBsAg, antibodies to appropriate viruses, bacteria, and fungi, and blood group alloantibodies. The concentrations of total protein, immunoglobulin, and albumin were determined, as were pH and conductivity. The pool was stored at 4 °C overnight and then fractionated.

Adsorption with Silicon Dioxide

Sterile, pyrogen-free, synthetic non-crystalline silicon dioxide (Aerosil 380, Degussa, Teterboro, New Jersey), in either wet or dry form, was added to the pooled plasma at a final concentration of 20–40 grams/liter. The suspension was stirred with motor-driven rotors for one hour at room temperature. Care was taken to avoid foaming. Following mixing, the silicon dioxide and its adsorbed proteins were sedimented at 6000 *g*.

Silicon dioxide adsorption of plasma has been found to remove, or to reduce the concentration of HBsAg (17), zymogens (18), pyrogens (19), and a number of labile plasma proteins (20). Table 1 shows the results of this treatment in the present study. The initial plasma pool had normal levels of fibrinogen, Factor XII (Hageman factor), prekallikrein, and complement components; after silicon dioxide treatment, none of these factors was detectable. In addition, this treatment removed lipoproteins, HLA antigens, and clotting Factors V, VII, VIII and XI.

The concentrations of IgA and IgM were reduced; that of IgG remained essentially unchanged except for the IgG3 subclass, which was either removed or markedly decreased (Condie, R.M.; Wistar, R., manuscript in preparation). An extensive study of antibody activity showed that neither the titers nor the specific activities of a variety of antibodies were reduced by SiO_2 treatment (Condie, R.M. *et al.*, manuscript in preparation). This is illustrated in Table 2. Measurement of antibody titers in plasma before and after adsorption with SiO_2 showed no change in antibodies to typhoid O and H antigens, in antistreptolysin O or anti-Candida activity, or in blood group alloantibodies.

Ion Exchange Chromatography

After adsorption with SiO_2, the plasma was sub-

Table 1. Removal of the Complement, Coagulation and Kinin Components of Plasma with SiO_2

	Concentration[a]	
	Plasma	SiO_2-treated Plasma
Total protein	64 mg/ml	70 mg/ml
Fibrinogen	300	0
Plasminogen	12	0
β-lipoproteins	420	0
HDL-associated HLA antigens	Present	Not detected
Cholesterol	160	0
Triacylglycerols ("triglycerides")	100	50
Clotting Factors	Percent Normal Activity	
II	100	100
V	100	0
VII	100	0
VIII	100	0
XI	100	0
XII (Hageman Factor)	100	0
Hemolytic C (screen)	Normal	No lysis
Hemolytic C (quant.)	24 units/ml	Not detected
C4	480	<10
C3	1200	<30
C1q	194	Not detected
C5	112	Not detected
Properdin	18.6	Not detected
C3PA[b]	187	Not detected
Prekallikrein	84[c] μmole/h-ml	0

[a] Unless other units are given, results of chemical analyses are expressed in milligrams per deciliter and concentrations of complement components are expressed in milligrams per liter.
[b] C3 Proactivator.
[c] Measured after kaolin activation.

jected to anion exchange chromatography. This was performed in four 16-liter bed capacity stacks, each containing ~650 g QAE-A50 Sephadex swelled in pH 7.0 imidazole-acetate sterilizing buffer. The stacks, the ion exchanger, and all the tubing were also sterilized in this buffer. Just prior to use, the sterilizing buffer was washed out with a starting buffer which had been filtered through a 0.2 μm (pore size) filter. The stacks were arranged in parallel flo with the flow rate kept constant at 150 ml/min through each stack. The adsorbed plasma, previously diafiltered to the starting conditions of pH and conductivity (see Fig. 1), was centrifuged at 6,500 *g* for 30 min at 22 °C and then applied to the top of the stacks. Samples were collected in sterile 4-liter graduated cylinders. The eluate was monitored by the absorbance at 280 nm. After the entire plasma pool had been applied, the protein not bound to the gel (i.e., IgG) was eluted with sterile pH 7.0 imidazole-acetate buffer. The protein was pooled into a sterile container and stored at 4 °C until final concentration, dialysis, and bottling. A typical elution profile is illustrated in Figure 1. Under proper conditions of pH and conductivity, the only protein to emerge in the first peak was IgG. The yields averaged >80% of the total IgG applied to the column. Albumin can easily be recovered in yields of >85% by lowering the pH, and can be further purified by passage over cation exchange columns.

Table 2. Specific Antibody Activity of Native IgG

	Antibody Titer		
	Plasma Pool	Post-SiO_2 Plasma Pool	Isolated IgG (10 mg/ml)
Typhoid O	512	512	512
Typhoid H	256	256	1,024
ASO[a]	512	256	1,024
Candida	1,024	4,096	10,240
Anti-A	8	4	negative
Anti-B	4	8	4
Herpes simplex	NT[b]	NT	256 IFA[c]
Varicella	NT	NT	64 IFA
Rubella	NT	NT	256 HI[d]
CMV	NT	NT	256 IHA[e]
HBsAg	negative	negative	negative
Anti-HBs	NT	NT	positive
			non-pyrogenic sterile

[a] ASO—antistreptolysin O.
[b] NT—not tested.
[c] IFA—indirect immunofluorescence.
[d] HI—hemagglutination inhibition.
[e] IHA—indirect hemagglutination.

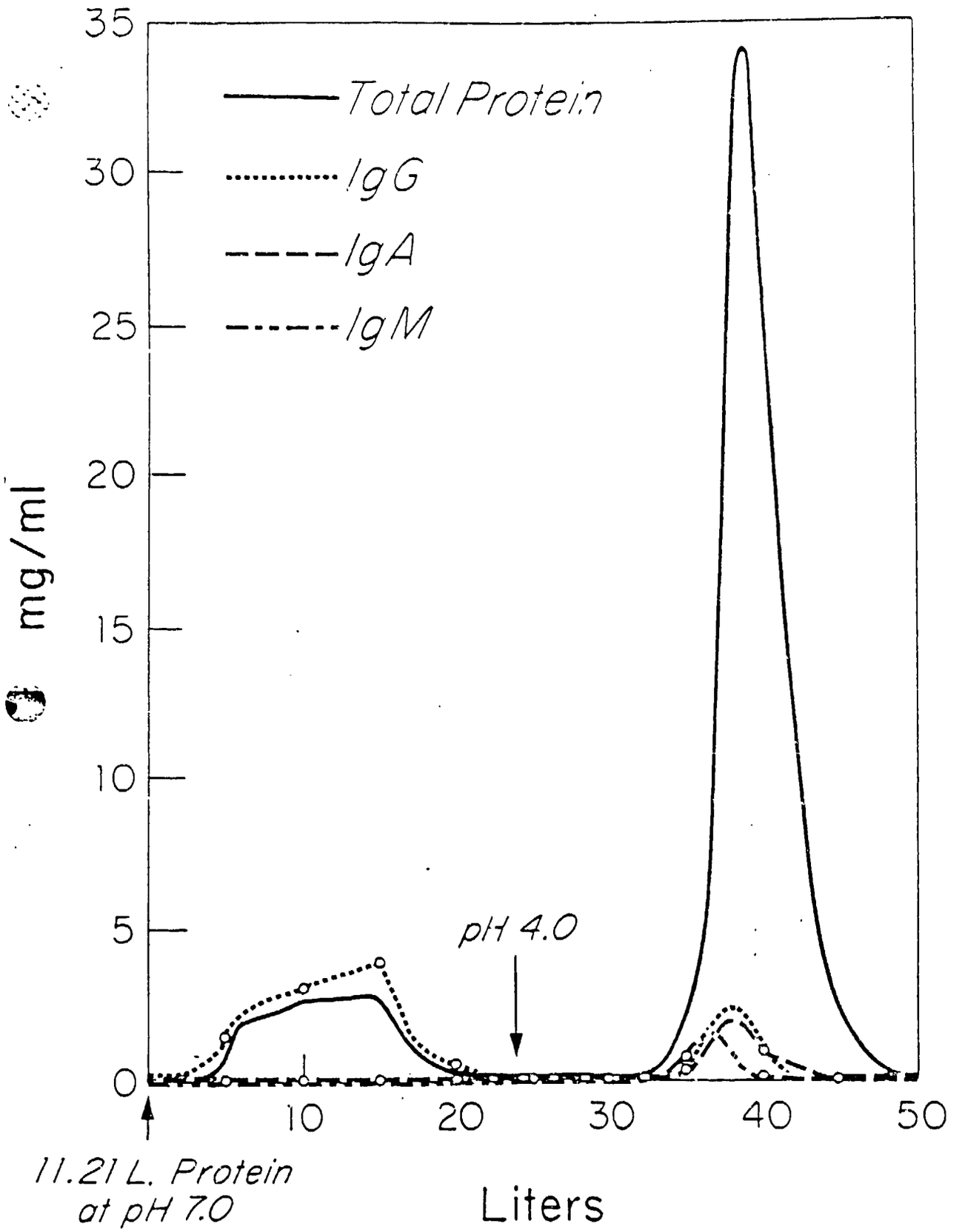

Figure 1. Chromatographic isolation of human IgG. Pooled plasma which had been adsorbed with SiO_2 was chromatographed on stacks of QAE-A50 Sephadex at pH 7.0 in imidazole-acetate buffer (0.15 M imidazole, 0.09 M sodium acetate, 0.009 M acetic acid; conductivity, 6.15 ± 0.1 mS at 23 °C). Elution with pH 4 buffer (0.108 M sodium acetate, 0.43 M acetic acid; conductivity, 6.30 ± 0.1 mS) removed many proteins, including albumin.

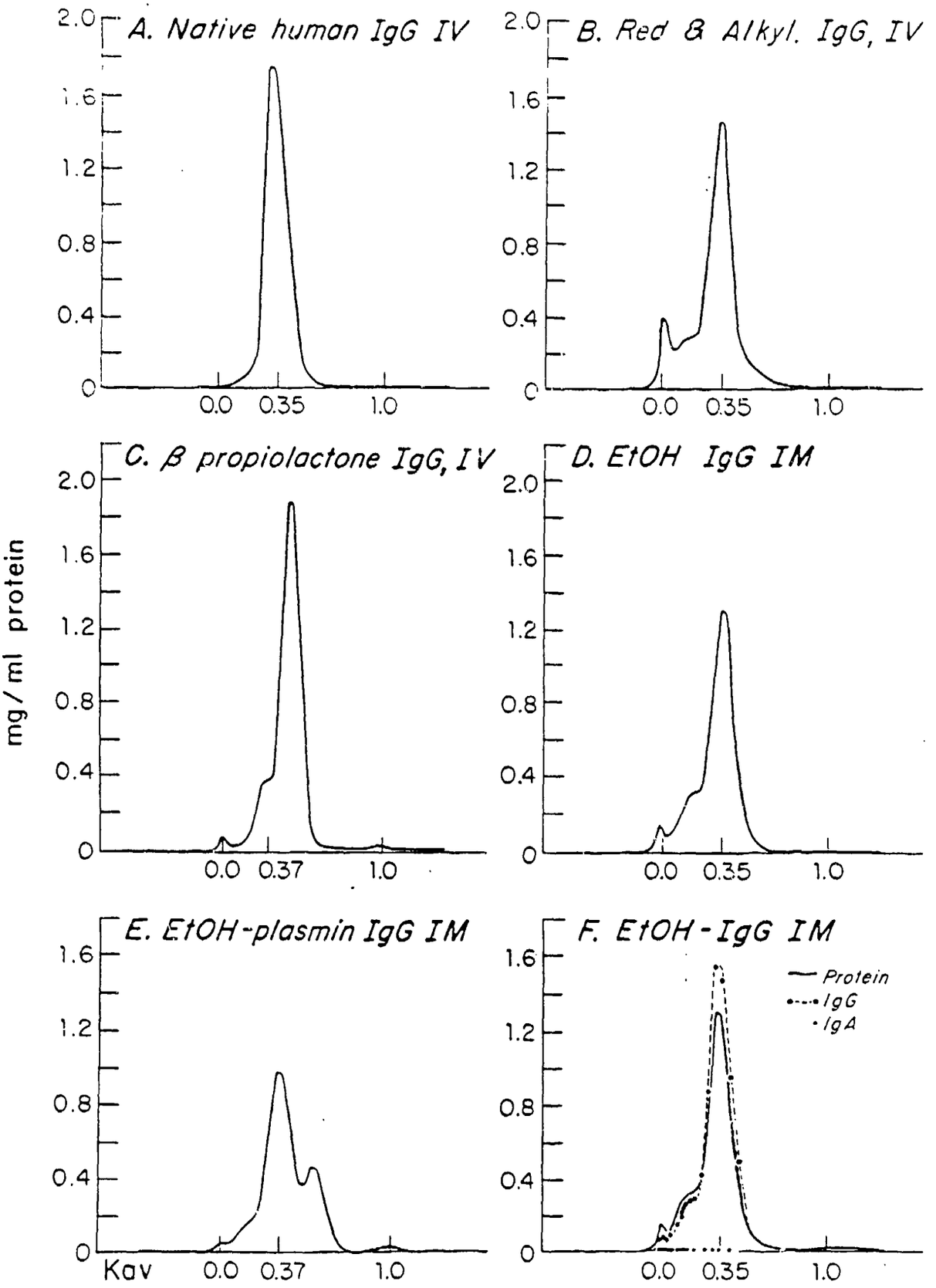

Figure 2. Measurement of aggregates and fragments of IgG by exclusion chromatography. Elution pattern shows the chromatographic behavior of various immunoglobulin preparations on a column of Biogel A-1.5m. Abscissa shows Kav.

Analytical Results: Comparison with Other IgG Preparations

Aggregation and Fragmentation

The immunoglobulin preparations isolated by the present method and by various other methods were analyzed for aggregation and fragmentation. Figure 2A shows the elution pattern of the present preparation during exclusion chromatography on Biogel A-1.5m. Neither aggregates nor fragments constituted as much as 1% of the total protein eluted. The elution profile and position were consistent with a monomeric IgG; accordingly, this preparation is referred to as native IgG.

Figure 2B is a representative elution curve of human IgG prepared by ethanol fractionation followed by reduction and alkylation. The major peak was not symmetrical. Aggregates constituted 20% of the total protein, and a considerable portion of these aggregates had molecular weights of >1,000,000.

Figure 2C is a protein elution curve of human IgG treated with β-propiolactone. This IgG contained more than 14% aggregates, some of which had very high molecular weight. It also contained detectable levels (2.5%) of IgG fragments (50,000 molecular weight). The main peak comprised only 83% of the total protein.

Figure 2D is a representative elution curve characteristic of IgG prepared by methods involving ethyl alcohol precipitation, polyethylene glycol, and salt precipitation. The protein elution curve was not symmetrical. Sixteen percent of the total protein comprised high molecular weight aggregates. This included dimers, trimers, tetramers, and aggregates with >1,000,000 molecular weight. There were no appreciable quantities of fragmented IgG.

Figure 2E is an elution curve from an ethanol-fractionated IgG which was degraded by plasmin. Although the amount of aggregates was reduced, 27% of the total protein consisted of IgG fragments with a molecular weight of ~52,000. Intravenous immunoglobulins have been prepared by treating alcohol-fractionated IgG with plasmin. However, the degree of proteolysis is difficult to control, and fragments of the plasmin-degraded material may be cleared very rapidly from the circulation (21,22).

Figure 2F represents the elution profile of an ethanol-precipitated IgG preparation analyzed with respect to total protein, IgG, IgA, and IgM. The high molecular weight aggregates, as well as the oligomers, were all composed of IgG.

Table 3. Comparison of the Molecular Size Distribution of Native IgG with that of Other Human IgG Preparations*

Preparation	Aggregates >300,000 MW (%)	Monomers 160,000 MW (%)	Fragments 50,000 MW (%)
Native human IgG IV	<1	99	<1
EtOH IgG IM	16	84	<1
EtOH plasmin IgG IM	7	63	27
Reduced/alkylated IgG IV	20	80	<1
β-propiolactone IgG IV	14	83	2.5

* Determined by exclusion chromatography. In each sample protein concentration was 45 mg/ml, the total volume 2 ml, and the total protein 90 mg. Each sample was applied to a 2.6 × 94 cm Biogel A-1.5m column.

Precipitation of IgG with ethanol, chemical modification with β-propiolactone, and reduction and alkylation all resulted in significant quantitites of aggregates (Table 3).

Isoelectric Focusing

The isoelectric range of the immunoglobulin preparations examined is presented in Table 4. The native IgG sample, prepared by the method described above, showed an isoelectric point (pI) range of 6.4–8.8. The β-propiolactone-treated IgG, with a pI range of 5.6–7.2, exhibited a significant chemical modification of surface charge. β-Propiolactone reacts with carboxyl, amino, hydroxyl, sulphydryl, and phenolic groups of proteins (23). Ethanol-precipitated IgG had a somewhat narrower range than the native material. However, the ethanol- and plasmin-treated IgG had the broadest pI range (6.18–9.43).

The thin-layer polyacrylamide gel (Fig. 3) from which these ranges were derived demonstrates another feature of these immunoglobulin preparations, namely, the macroheterogeneity. The two procedures which introduced significant macroh-

Table 4. Isoelectric Range of Various Human IgG Preparations*

Preparation	pI Range
EtOH IgG IM_1	6.7–8.7
EtOH IgG IM_2	7.0–8.8
EtOH plasmin IgG IM	6.18–9.43
Native human IgG IV	6.4–8.8
Reduced/alkylated IgG IV	6.5–9.0
β-Propiolactone IgG IV	5.6–7.2

* Determined by thin-layer isoelectric focusing in polyacrylamide gels. In each sample protein concentration was 5.0 mg/ml, the total volume 20 µl, and the total protein 0.1 mg.

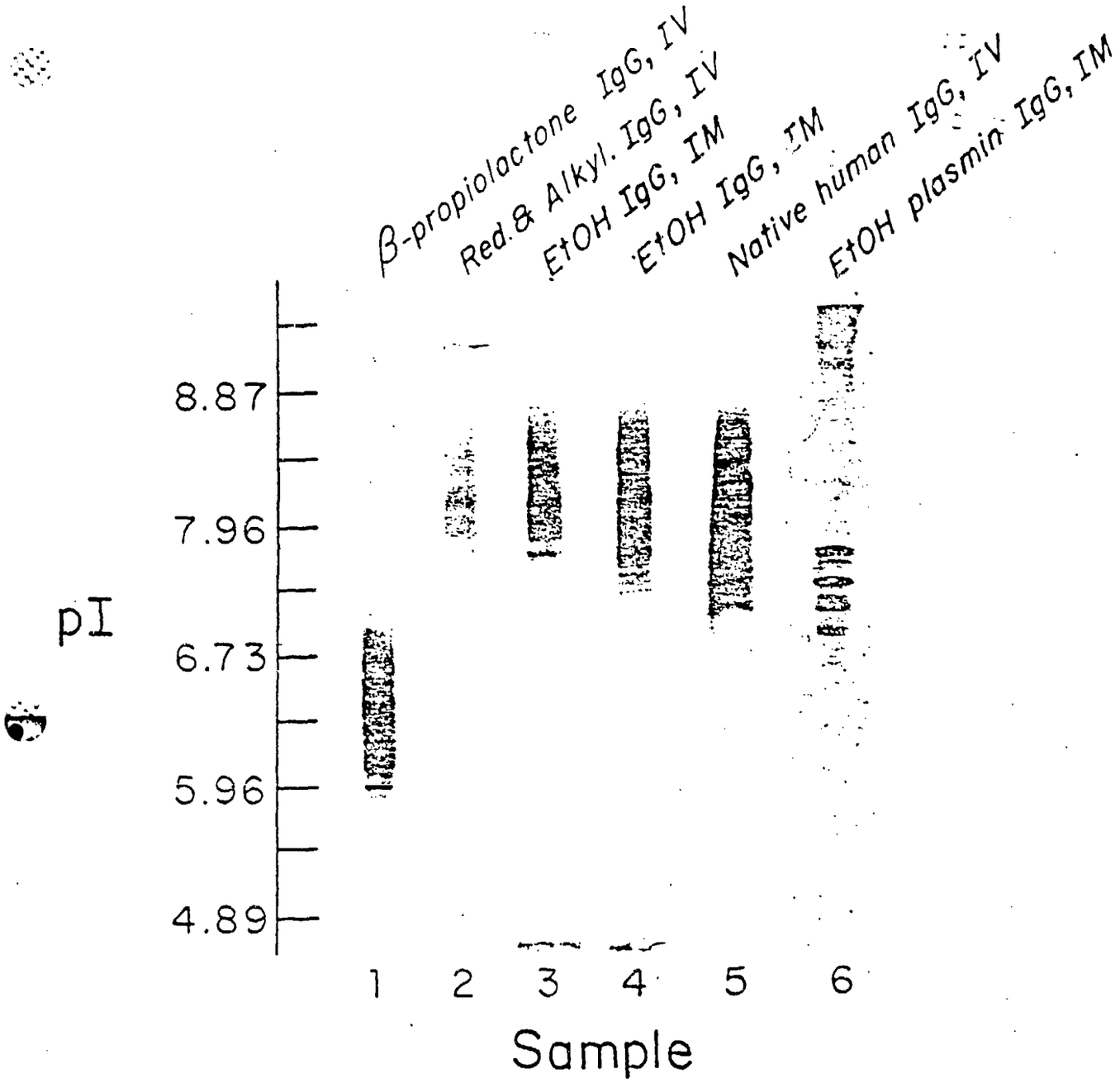

Figure 3. Thin-layer isoelectric focusing of various immunoglobulin preparations in polyacrylamide gel. The pH gradient and the preparations analyzed are indicated on the figure. Samples containing 0.1 mg protein were focused for a total of 100 min and stained with Coomassie Blue.

eterogeneity were the β-propiolactone treatment and plasmin digestion. The sharp dense bands in the plasmin-treated sample were derived from fragmentation of aggregates and degradation of some of the monomeric molecules. The β-propiolactone-treated IgG demonstrated the most marked alterations, leading one to expect an impairment of some effector functions of the molecule (4,5). In addition, alteration of surface charges indicates that native groups have been replaced by new chemical groupings which might act as new antigenic determinants capable of generating antibody which could react with the β-propiolactone-treated molecule (24).

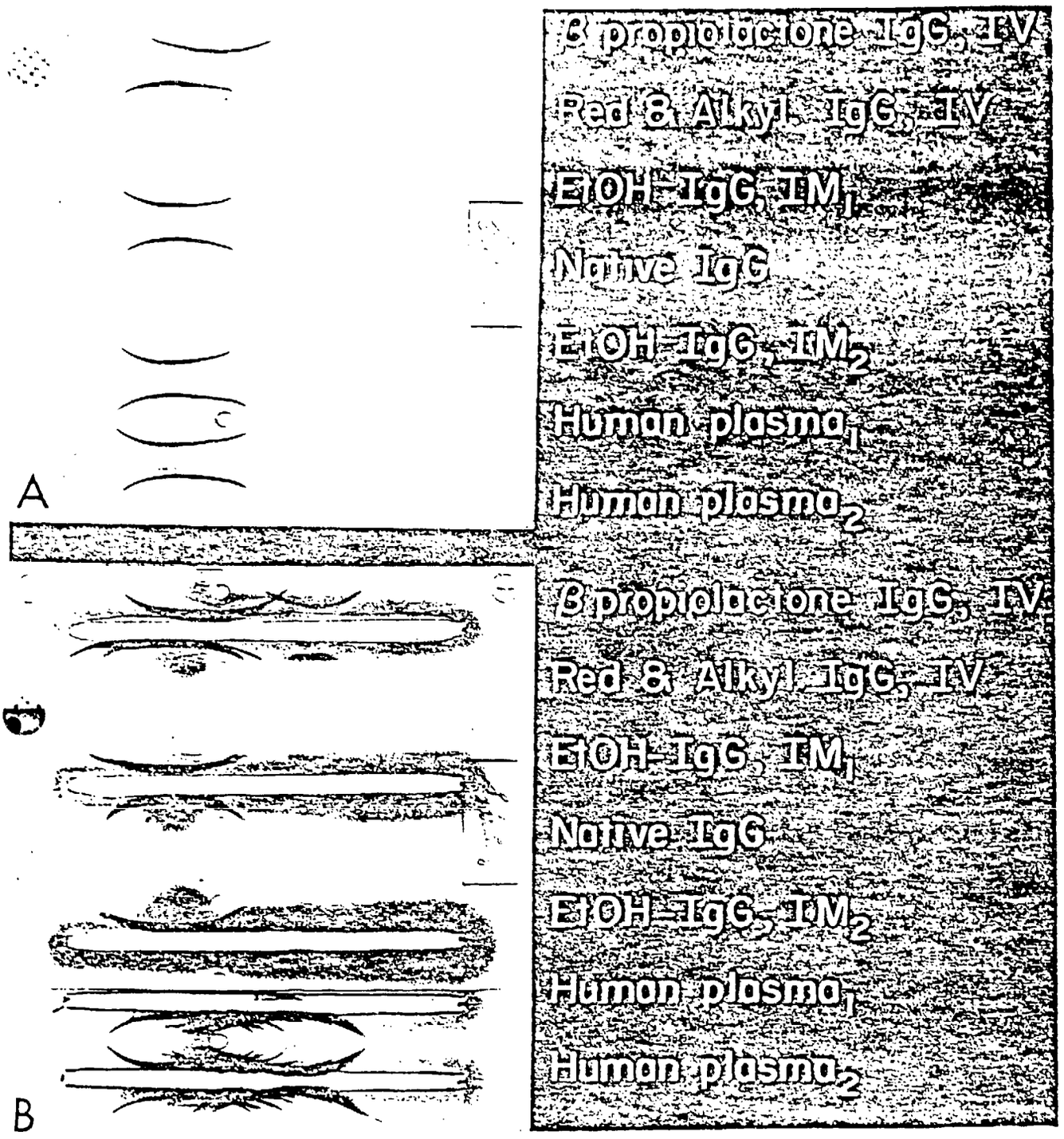

Figure 4. Immunoelectrophoresis of various immunoglobulin preparations. The electrodes and the preparations analyzed are indicated on the figure. Samples (40 mg protein/ml) were subjected to 150 V for 80 min and allowed to diffuse against antiserum for 24 h. Plates were washed for 48 h and stained with Amido Black. Plate A troughs contained anti-human IgG (γ-chain specific); plate B, anti-whole human serum.

Immunoelectrophoresis

Figure 4 shows the relative electrophoretic mobilities of the IgG, as well as the impurities, in various IgG preparations. As expected from the results of isoelectric focusing (Fig. 3), β-propiolactone-treated IgG exhibited clearly altered electrophoretic mobility (Fig. 4A). The other preparations showed electrophoretic mobilities similar to that of IgG in plasma.

To demonstrate the presence of other proteins

Table 5. Comparison of Anti-Complementary Activity (C1 Binding) of Five Human IgG Preparations

Preparation	Percent Monomer	Percent Aggregation	Anti-Complementary Activity[a]
Native IgG IV	99	<1	50.00
EtOH IgG Red Cross IM	69	30	0.15
EtOH IgG IM	84	16	0.44
EtOH-Reduced/alkylated IgG IV	80	20	8.00
EtOH-plasmin IgG IM	63[b]	7	22.00

[a] Micrograms of protein required to fix 50% C1

[b] Fragment (52,000 MW) comprised 27% of this preparation.

in these preparations, immunoelectrophoresis was performed with anti-whole human serum (Fig. 4B). This experiment showed that the β-propiolactone-treated IgG contained two major components and at least two other proteins that formed arcs near the well. One ethanol-precipitated preparation contained a small quantity of an anodal component and two minor components that appeared as arcs near the well; moreover, it showed some splitting of the major IgG arc. A second ethanol-precipitated immunoglobulin contained the two minor components, but exhibited neither the anodal component nor splitting of the IgG arc. The native IgG appeared essentially homogeneous by this technique; in addition to the IgG, only a single minor arc was observed.

Anti-Complementary Activity

In its normal state in blood, IgG does not interact appreciably with components of the complement system. The biologically active sites within the Fc region of IgG that interact with complement are expressed or exposed as a result of conformational or structural changes. Such changes normally occur following the combination of a specific antibody with surface antigens on bacteria, viruses, or altered cells. This combination mediates many aspects of inflammation and facilitates ingestion of pathogens by phagocytes. Once the site in the Fc region is exposed, the first component of complement (C1) attaches through a non-covalent linkage or ionic bond, which results in enzymic activation of the complement cascade. Structural alterations (such as aggregate formation) also expose these biologically active complement-fixing sites on the IgG molecule. Therefore, the extent of complement fixation or anti-complementary activity of IgG can be used as a measurement of denaturation and alteration from the native state.

It was observed in 1944 (25) that fractionated IgG was anti-complementary. Subsequently, it was demonstrated that aggregates in the fractionated IgG were responsible for this effect (26). Later, in 1959, it was shown that heat-denatured (aggregated) IgG fixed complement in a manner indistinguishable from that of antigen-antibody complexes (27).

In our studies, the comparison of anti-complementary activity of various IgG preparations was made by determining the number of micrograms of IgG protein required to fix 50% of a standard preparation of complement component C1 (28). In general, there was a direct relationship between the extent of aggregation and the anti-complementary activity (Table 5). The only exception to this direct relationship was shown by the reduced and alkylated IgG. Although its aggregate content was similar to that of the ethanol-precipitated preparations, its complement-fixing activity was less than one-seventeenth as great (Table 5). Thus the reduction and alkylation process evidently decreased the anti-complementary activity. Similarly, plasmin digestion and β-propiolactone treatment reduce the anti-complementary activity of immunoglobulin preparations, even though appreciable quantities of aggregates produced by the fractionation process may be present. Complement fixation therefore appears to have real limitations as a tool for assessing the denaturation of IgG.

Susceptibility to Limited Proteolysis

The unfolding of native proteins makes peptide bonds more accessible to enzymic action; hence, one would expect denatured proteins to be more susceptible than native proteins to proteolysis by enzymes such as plasmin. To assess the extent of denaturation, the present IgG preparation and immunoglobulins prepared by other methods were therefore exposed to plasmin. The effects of this exposure to plasmin on the behavior of IgG during exclusion chromatography are illustrated in Figure 5. Native IgG, incubated at 37 °C for 18 h without

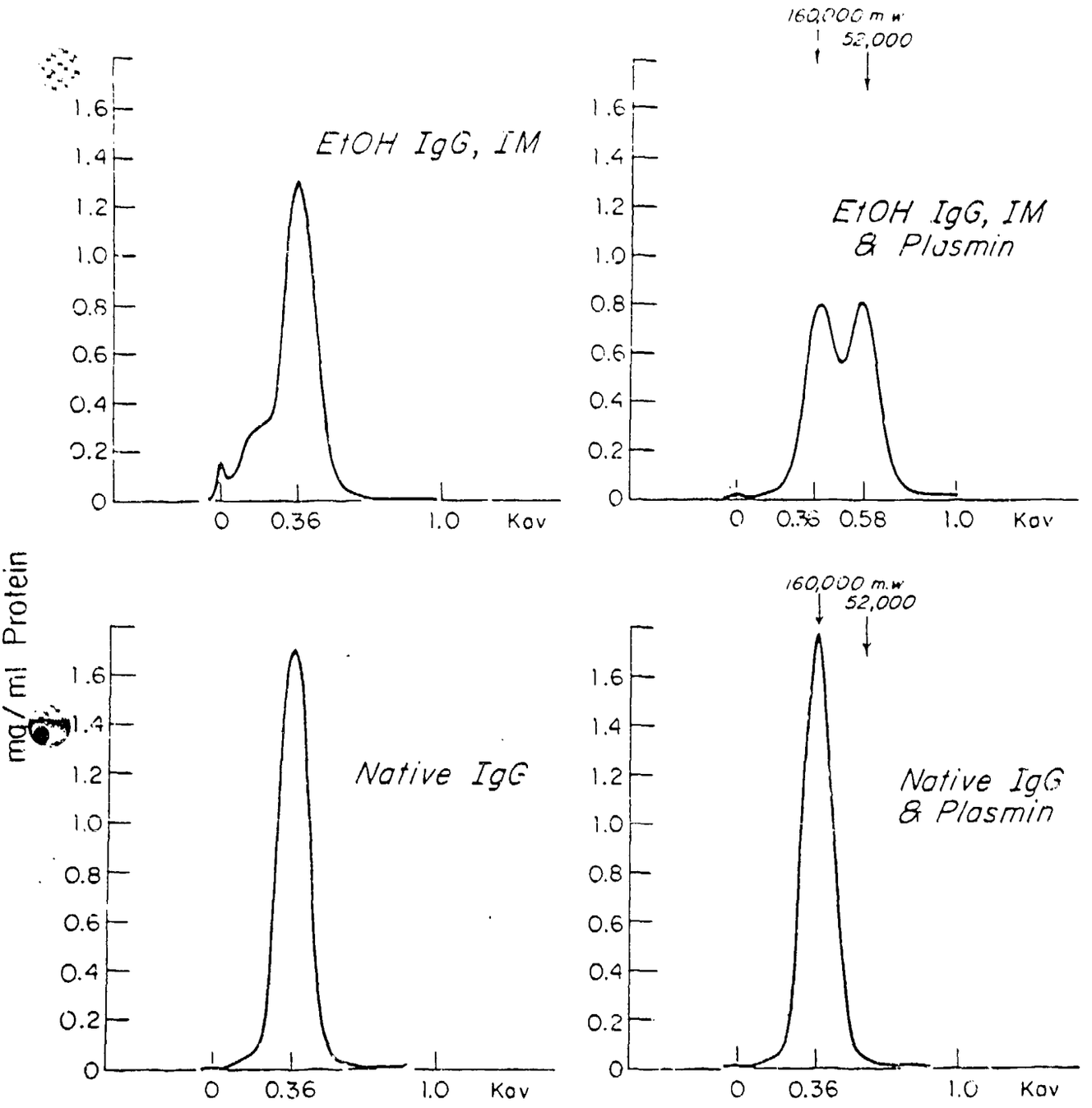

Figure 5. Effect of plasmin treatment on native IV IgG and ethanol-precipitated IM IgG. Elution patterns show the chromatographic behavior of immunoglobulins on Biogel A-1.5m (see Fig. 2) after incubation at 37 °C for 18 h in the absence (left) or presence (right) of plasmin.

plasmin, was eluted from the Biogel column in a symmetrical peak that corresponded to a molecular weight of 160,000 (cf. Fig. 2A). Following the treatment with plasmin, the native IgG appeared unaltered. In contrast, the elution pattern of an ethanol-precipitated (IM) immunoglobulin preparation incubated at 37 °C for 18 h with no enzyme showed extensive aggregation, including oligomers and high-molecular aggregates. Incubation of this preparation with plasmin under the identical conditions as the native IgG resulted in the disappearance of most aggregates, reduction of the main

Table 6. Differential Effect of Plasmin on EtOH IM IgG and Native IgG

Sample	Percent[a] Aggregates	Percent in 160,000 Peak	Percent in 52,000 Peak
EtOH IgG IM	13	87	<1
EtOH IgG IM + Plasmin	1	48	51
Percent 160,000 peak degraded by plasmin		39[b]	
Native IgG	<1	99	0
Native IgG + Plasmin	0	98	1
Percent 160,000 peak degraded by plasmin		1[b]	

[a] Values determined by planimetry of elution curves obtained by exclusion chromatography on Biogel A-1.5m (see Fig. 5).
[b] Determined by difference.

(160,000 molecular weight) peak, and formation of a second peak containing 52,000 molecular weight fragments of IgG.

Quantitation of the differential effect of plasmin on ethanol-precipitated (EtOH IM) and native IgG is presented in Table 6. Not only the aggregates, but also the main (160,000 molecular weight) component of the former was sensitive to plasmic proteolysis. At least 39% of the monomeric IgG was hydrolyzed and appeared as 52,000 molecular weight fragments. Other methods of preparing IgG, including β-propiolactone treatment, reduction and alkylation, salting out, and polyethlene glycol precipitation, render the protein susceptible to attack by plasmin. The limited proteolytic assay appears to provide a more direct assessment of IgG denaturation than does the complement fixation test.

Prekallikrein Activator Activity

It has been proposed that the flushing, erythema, and hypotension associated with the IV infusion of Plasma Protein Fraction (PPF) under certain conditions result from the presence of Hageman-factor fragments (prekallikrein activator, PKA) activated during ethanol fractionation of plasma (11). The present preparation is free of this contaminant, whereas IM ethanol-precipitated immunoglobulins can contain high levels of PKA.

Circulatory Half-Life

Intravenous injection of fresh human plasma is generally safe and not associated with adverse reactions. The IgG present in fresh plasma has a half-life of 24–32 days. Therefore, a native IgG preparation should be expected to possess similar

Table 7. Circulatory Half-Life of Various Human IgG Preparations Following Intravenous Administration

Preparation	Half-Life[a]
Native IgG	18–28
Fresh plasma (IgG)	24–32
EtOH plasmin IgG	
Intact IgG	18–20
Fc fragment	8–10
Fab fragment	5 h
β-Propiolactone IgG	4–12

[a] Expressed in days unless other unit given.

characteristics upon intravenous administration. Table 7 includes the circulatory half-lives of several IgG preparations. The present preparation had a half-life of 18–28 days. The other preparations showed significantly reduced half-lives, ranging from 5 h for the Fab fragments in the plasmin-treated product to 18–20 days for its monomeric IgG component. β-Propiolactone-treated IgG is reported to have a half-life of 4–12 days (4,5).

Clinical Use and Adverse Reactions

Depending on the patient population, adverse reactions to the IV administration of immunoglobulin can vary, e.g., from 93% in patients with an immunodeficiency syndrome to 13% in normal, healthy individuals (2). Normal plasma does not produce reactions in either group (Table 8). β-Propiolactone treatment of IgG significantly reduces the incidence of severe reactions (from 93% to 15%) in immunodeficient patients (5). Native IgG, however, causes an even lower reaction rate (<1%).

Table 8. Incidence of Severe Reactions to Intravenous Administration of Various IgG Preparations

Preparation	Number of Patients	Incidence of Reactions	Reference
Normal Fresh Plasma			
Immune deficiency syndrome patients	6	none	Kirkpatrick C. H. (This volume)
Native IgG			
Patients with life-threatening infections	107	<1%	Present study
EtOH IgG			
Normal, healthy persons	55	13%	2
Immune deficiency syndrome patients	15	93%	2
β-Propiolactone IgG			
Immune deficiency syndrome patients	12	15%	5

Table 9. Clinical Use of Human Intravenous Immunoglobulin[a]

	Total Number
Patients treated	167
IV administrations (20–200 mg/kg)	896
Grams protein administered	3685
Side effects	
Serious reactions	0
Mild reactions	5[b]
HBsAg conversions	1

[a] The IgG was prepared by chromatography (QAE–A50 Sephadex) of SiO_2-treated plasma. Starting material: Red Cross outdated and cryoprecipitate-poor plasma from volunteer donors. Total quantity of IgG prepared: 4715 grams. Time span: December 20, 1974–June 20, 1979.
[b] Reactions reported: flush, headache alone, and headache with chills.

The total clinical experience with patients who have been followed for at least six months after they received IV IgG is summarized in Table 9. During the past four and one-half years, 167 patients received IV IgG in doses ranging from 20 to 200 mg/kg. Problems associated with administration of this preparation have been minimal. In a total of 896 separate IV administrations, five reactions occurred. These consisted of flushing, headache and chills. The single patient in whom there was HBsAg conversion received more than 40 units of blood during a long and stormy clinical course.

DISCUSSION

In the past, most attempts to prepare an immunoglobulin that would be safe for IV use have involved the addition of a stabilizer (e.g., sucrose, reducing sugar, albumin) or modification of the product after fractionation. The rationale for the present effort to prepare a native, undenatured (rather than a modified) IgG stems from the view that preserving the normal structural integrity of this complex molecule will result in a stable, safe and more effective product.

Over the years instability has been a consistent troublesome characteristic of purified human immunoglobulins, suggesting that these proteins are either inherently unstable or irreversibly altered during fractionation and that these properties are somehow responsible for the adverse reactions produced by intravenous administration.

More careful examination of the problem points to a complex set of conditions, possibly involving trace contamination by PKA activity generated from labile plasma protein precursors as well as alteration of the structures within the IgG molecule that control its biologic effector functions. This alteration takes the form of aggregate formation and spontaneous cleavage of IgG during storage, leading ultimately to reduced biologic effectiveness. Evidence that isolated IgG preparations undergo degradative changes during storage in solution was first denoted by a splitting of the precipitin arc during immunoelectrophoresis (29). Later studies showed that during storage at 4 °C dimerization of up to 20% of the IgG molecules could occur; this was accompanied by increased vulnerability to proteolysis by contaminating enzymes (30). In IgG isolated from plasma by the Cohn-Oncley method the contaminating enzyme was plasmin, and the level of fragments (3.5 S Fab and Fc) formed during storage could reach 60% of the total protein (31).

The biologic effectiveness of aged, fragmented preparations of human IgG was shown to be impaired (when compared to fresh, unfragmented material) by their decreased ability to induce passive protection in guinea pigs against challenge with tetanus toxin and in monkeys against poliomyelitis virus Type I (3). The significantly reduced biologic effectiveness was apparently the result of both a reduction in the number of intact neutralizing antibody molecules and the marked reduction in circulatory half-life that occurs when the Fab fragments are cleaved away from the Fc domain, which regulates the circulation time of IgG (32,33).

The present procedure for isolating IgG circumvents this type of degradation problem. In this procedure plasminogen and plasmin are removed during the first step by adsorption of the plasma with SiO_2.

Even if the cold-ethanol fractionation method could routinely yield IgG preparations that were free of enzymic activity, the aggregation of IgG that occurs during this process would continue to present a major problem. The extreme susceptibility of immunoglobulins (in contrast to other plasma proteins) to the denaturing action of ethanol was recognized by Cohn (7). Control of the same five variables (temperature, ionic strength, pH, ethanol and protein concentrations) that were shown to determine the purity and yield of each protein isolated (7) also determines the extent of denaturation of these proteins (Condie, R.M.; Bethel, G.U., manuscript in preparation).

The denatured IgG molecule exhibits a number of altered biologic effector functions. Although

some alterations from the native state can be demonstrated by structural studies and other tests performed *in vitro*, behavior of such IgG in the circulation can only be assessed by studies *in vivo*. Thus the final definition of the native molecule must comprise both structural and biological evidence. The former includes electrophoretic mobility, isoelectric point(s), molecular weight (freedom from aggregates and fragments), anticomplementary activity (low), and resistance to proteolysis. The biological indicators include circulatory half-life (and absence of rapidly-cleared components), safety for intravenous administration in large quantities (200 mg/kg), and freedom from components that activate the complement, coagulation, or kallikrein-kinin system.

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DISCUSSION

DR. STITES: What are the efficacy data for your preparation with respect to treatment of renal transplant patients who have CMV?

DR. CONDIE: Seventy percent of the patients treated with the immunoglobulin preparation in high doses recovered, and they are currently alive and healthy. Thirty percent of the patients died as a result of CMV infection. There is an indication in the study that the earlier the treatment is initiated, the better will be the results. In the first part of the study this treatment was used as a last resort, and we were treating essentially dead patients. If CMV can be diagnosed early in its course then we think that the therapy is efficacious, but we have not done a controlled trial.

DR. EKRE: How do you store your preparation, and do you have data on its stability?

DR. CONDIE: We stored it two ways. We used to freeze it, but we became concerned because the top layer on prolonged freezing seems to dry out. We now store it at 4 °C. We have samples that have been at room temperature for two years, and we have periodically tested for aggregates and found little evidence that either aggregation or fragmentation occurs in our preparation during this time. In fact, we have even stored plasma for several years, after the silica treatment. No changes have been detected, provided the solution is sterile.

DR. FINLAYSON: What is the solvent for your material as you store it?

DR. CONDIE: It is 0.3 M glycine.

DR. COVAL: Have you reported any specific antibody levels?

DR. CONDIE: Our preparation, at a protein concentration of 10 mg/ml, has an anti-CMV titer of 256 by immune fluorescent assay. This is about a 3- to 5-fold increase of specific antibody activity over starting plasma. I think for every method one has to determine whether one is isolating the active antibodies. There may be some antibodies in human plasma that are not in the major classes that we are isolating. I do not know if there are any antibodies that are only in the IgG3 subclass, but if there are, they will not be in our preparation.

DR. NEUMANN: If you apply this procedure to plasma known to contain HBsAg, is the antigen removed by the procedure?

DR. CONDIE: It has been reported that it is. We have not tested this.

DR. KORNHUBER: You reported that severe side effects occurred in 12 percent of the patients with immunodeficiency who received β-propiolactone-treated immunoglobulin.

DR. CONDIE: These studies were reported in the literature in the early 1970's, when the β-propiolactone-treated immunoglobulin was first being prepared.

DR. KORNHUBER: We have only three patients with Bruton-type immunodeficiency, but we have not seen any side effects in these patients after administration of the β-propiolactone-treated immunoglobulin. One of them has received more than 400 grams of immunoglobulin during the past 10 years, and he has had no side effects.

DR. STEPHAN: The half-life of the β-propiolactone-treated immune globulin in healthy people is between 15 and 21 days; the half-life you reported was determined in people who were not healthy.

Can proteins other than IgG be fractionated by your method?

DR. CONDIE: Yes, there is an excellent albumin recovery, although we are not preparing albumin. We get between 85 and 90 percent recovery of albumin from the plasma applied to the column. We are not recovering albumin routinely because we have no need for it at the present time.

DR. STEPHAN: Your stabilization process, which uses Aerosil (silicon dioxide), was published by our group (Biotest) in 1968; I feel one should mention this.

DR. CONDIE: Yes, our patent attorneys uncovered that. We were unaware of this at the time we started.

DR. FINLAYSON: Perhaps I should mention that Biotest should have pointed this out in view of the fact that this material has been referred to as the β-propiolactone-treated material for many years when, in fact, as Dr. Stephan has correctly said, it was the β-propiolactone and, also, the Aerosil-treated material.

HUMAN GAMMA GLOBULIN PRODUCTION CHECKLIST

LOT # IVBG-1A

Note: a) Letters and numbers in parentheses at left refer to steps in Protocol F3.

b) Place date and your initials in underlined space to right when procedure completed.

c) P = protein concentration in mg/ml; TP = total protein, in grams.

(A2) HUMAN CRYOPOOR PLASMA:

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072354		Mich	3/21/79	072600		Mich	4/26/79
072381			3/21/79	072998			7/3/79
072456			4/5/79	072999			7/3/79
072254			3/7/79	073181			9/11/79
072454			4/5/79	072315			3/15/79
072130	N-O		7/26/79	072151			7/31/81
072988			7/3/79	072057			7/12/79
072992	N-O		7/3/79	073107			8/30/79
072990			7/5/79	072182	N-O		8/2/79
072127	N-O		7/24/79	072999			7/3/79
072990			7/3/79	073182			9/11/79
072300			3/13/79	072290			3/13/79
073047	N-O		8/21/79	073098	N-O		2/28/79
072598			4/26/79	072060			7/12/79
072055			7/12/79	072996	N-O		7/3/79
072129			7/26/79	072295			3/13/79
072129			7/26/79	073250			9/25/79
072984			7/3/79	073291			9/27/79
072986			7/3/79	073226	N-O		9/18/79
072213	N-O		8/7/79	072303			3/13/79
072055			7/12/79	073243			9/20/79
072984			7/3/79	073336	N-O		10/4/79
072181	N-O		8/29/79	073296			10/2/79
072060			7/12/79	072283	N-O		3/13/79
072183			8/2/79	073065			8/23/79
073108		V	8/30/79	073083			8/28/79
072277			3/8/79	073117	N-O		8/30/79
072181	N-O		8/2/79	072297		V	3/13/79
072998			7/3/79	072603			4/26/79
072994			7/3/79	073114			8/30/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073238		[illegible]	9/20/79	073109		mich	8/30/79
073065		mich	8/23/79	073247	N-0		9/20/79
073244			9/26/79	072976	N-0		7/3/79
073111			8/30/79	073215	N-0		9/18/79
073239	N-0		9/20/79	073217			9/18/79
073195			9/13/79	073272			9/18/79
073038			8/21/79	073026			8/16/79
073041	N-0		8/21/79	072103	N-0		7/19/79
073032			8/16/79	073102	N-0		8/30/79
073196	N-0		9/13/79	073080			8/28/79
073243			9/20/79	072201			8/7/79
073058			8/23/79	072076	N-0		7/17/79
073082			8/28/79	073022			7/16/79
072997			7/3/79	072076			7/17/79
073238			9/20/79	073215	N-0		9/18/79
073118			8/30/79	072080			7/17/79
072466			4/5/79	073032			8/16/79
072736			5/17/79	072968	N-0		6/28/79
072515	N-0		4/12/79	072968	N-0		6/28/79
072301			3/13/79	072970			6/28/79
072300			3/13/79	073237			9/20/79
072964			6/28/79	073100			8/30/79
072276			3/8/79	073101			?
072127	N-0		7/24/79	072869			6/6/79
072442	N-0		4/3/79	072869			6/6/79
072414			3/29/79	072897	N-0		6/12/79
072281			3/13/79	073099			8/28/79
072598			4/26/79	073072			8/23/79
072452			4/5/79	072861			6/5/79
072361			3/21/79	073101			?
073247	N-0		9/20/79	073152			9/6/79
073110			8/30/81	073095	N-0		
073109			8/30/79	073154			9/6/79
073214			9/18/79	072896			6/12/79
073110			8/30/79	073100			8/30/81
072183			8/2/79	073073			8/23/79
072931			6/14/79	073153			9/16/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072983		Mich	6/28/79	072184		Mich	8/2/79
072861			6/5/79	073102	N-O		8/30/79
073153			9/16/79	072382			3/27/79
072822			5/30/79	072514			4/12/79
073070			8/23/79	073078			8/27/79
072821			5/29/79	073214			9/18/79
073154			9/16/79	072361			3/21/79
072925	N-O		6/14/79	072354			3/21/79
072926	N-O		6/28/79	072457			4/5/79
072925			6/14/79	072457			4/5/79
072848			6/1/79	072455			4/5/79
072848			6/1/79	072356	N-O		3/21/79
072818			5/29/79	072381	N-O		3/27/79
073074	N-O		8/23/79	072313	N-O		3/15/79
073155			9/6/79	072451	N-O		4/5/79
072846			6/1/79	072311			3/15/79
072818			5/29/79	072309			3/15/79
073155			9/6/79	072321			3/15/79
072931			6/14/79	072608			4/26/79
073113			8/30/79	072311			3/15/79
073117	N-O		8/30/79	072301	N-O		3/15/79
073213			9/18/79	072452			4/5/79
072966			6/28/79	072451	N-O		4/5/79
072139			7/26/79	072516			4/12/79
073239	N-O		9/20/79	072446			4/3/79
073244			9/20/79	073108			8/30/79
073030	N-O		8/16/79	072275			3/8/79
073250			8/25/79	072007			7/5/79
073162	N-O		8/30/79	072121	Non-O		7/24/79
073082	N-O		8/28/79	072109	N-O		7/24/79
073215	N-O		9/20/79	072117			7/24/79
073118	N-O		8/30/79	072156	N-O		7/31/79
072138			7/26/79	072004			7/3/79
073038			8/21/79	072180			8/2/79
073033	N-O		8/16/79	072083	N-O		7/17/79
072356	N-O		3/21/79	072074			7/17/79
072513			4/12/79	072184			8/7/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072079		Mich	7/17/79	073137	N-O	Mich	9/4/79
072002			7/3/79	072075			7/17/79
072034	N-O		7/10/79	072981			6/28/79
072035			7/10/79	072137			7/26/79
072032	N-O		7/16/79	072979			6/28/79
072080			7/17/79	072143			7/26/79
072077	N-O		7/17/79	072974			6/28/79
072034	N-O		7/10/79	072074			7/17/79
072196			8/7/79	072967			6/28/79
072036			7/10/79	072141	N-O		7/28/79
072138			7/26/79	072203			8/7/79
072075			7/17/79	072967			6/28/79
072103	N-O		7/19/79	072179			8/2/79
072078			7/17/79	073045			8/21/79
072035			7/10/79	073045			8/21/79
072081			7/17/79	072175	N-O		8/2/79
072189	N-O		8/7/79	073070			8/23/79
072189	N-O		8/7/79	073067			8/21/79
072147	N-O		7/26/79	073044			8/21/79
072191	N-O		8/2/79	073044			8/21/79
073187			9/13/79	072102			7/19/79
073188	N-O		9/13/79	073326			10/4/79
073124	N-O		9/4/79	072249			8/14/79
073183			9/13/79	073074	N-O		8/23/79
073190			9/13/79	073129			9/4/79
073127			9/4/79	073236			9/20/79
072198			8/7/79	072246	N-O		8/14/79
073189			9/13/79	072248			8/14/79
072192			8/2/79	073027			8/16/79
073189			9/13/79	072250			8/14/79
072186			8/7/79	073001			8/14/79
072036			7/10/79	072136			7/26/79
073187			9/13/79	072139			7/26/79
072195			8/7/79	072972			6/28/79
073087			8/20/79	073235			9/20/79
073021			8/16/79	072983			6/28/79
072143			7/26/79	073069			8/23/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072214	N-O	Mich	8/7/79	073087			8/28/79
072214	N-O		8/7/79	073088			8/28/79
072827			5/31/79	072137			7/26/79
073069			8/23/79	073024			8/16/79
073066			8/23/79	073024			8/16/79
072013			7/5/79	073130			9/14/79
072993			7/3/78	073137	A-O		9/4/79
072216	N-O		8/8/79	073089			8/28/79
072836			5/31/79	072966			6/28/79
072993			7/3/78	073127			9/4/79
072836			5/31/79	073124	A-O		9/4/79
073066			8/23/79	073088			8/28/79
073068	N-O		8/23/79	072023	N-O		7/5/79
073149			9/6/79	072166			7/3/79
073067			8/23/79	073087			8/28/79
072973			6/28/79	072424			4/3/79
072216	N-O		8/8/79	072406			3/29/79
072423			4/3/79	073021			8/16/79
072386	N-O		3/27/79	072533			4/17/79
073127			9/4/79	072533			4/17/79
072971	A-O		6/28/79	072125			7/24/79
072387			2/27/79	072161			7/31/79
072427			4/3/79	072828			5/31/79
073122			9/4/79	073134			9/4/79
072216	N-O		8/8/79	072351			3/22/79
072427			4/3/79	072404			3/29/79
073122			9/4/79	072059			7/12/79
072147	A-O		7/26/79	072191	N-O		8/2/79
073183			9/13/79	072063	N-O		7/12/79
072145			7/26/79	072088	N-O		7/19/79
072145			7/26/79	072163			7/31/79
072403			3/29/79	072023	N-O		7/5/79
072402	A-O		3/29/79	072164			7/31/79
072402	A-O		3/29/79	073180	N-O		9/11/79
072974			6/28/79	072120			7/24/79
073133	N-O		9/4/79	072084	N-O		7/17/79
				072154			7/31/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072082		MKL	7/17/79	072129	N-O	Mich	7/24/79
072121	N-O		7/24/79	073171	N-O		9/11/79
072156	N-O		7/31/79	072187	N-O		8/2/79
072221			8/9/79	072179			8/2/79
072159	N-O		7/31/79	072186			8/2/79
072088	N-O		7/19/79	073141			9/4/79
072126	N-O		7/24/79	072146	N-O		7/26/79
072005	N-O		7/3/79	072038			7/16/79
072005	N-O		7/3/79	073134			9/4/79
072163			7/31/79	072113			7/24/79
072219			8/9/79	073138			9/4/79
072224			8/9/79	073026			8/16/79
072973			6/28/79	073022			8/16/79
072975			6/28/79	073237			9/20/79
072978	N-O		6/28/79	073216			9/18/79
072978	N-O		6/28/79	073077	N-O		8/23/79
072188			8/2/79	072246	N-O		8/14/79
072972			6/28/79	073216			9/18/79
072185			8/2/79	073172			9/11/79
072187	N-O		8/2/79	073075	N-O		8/23/79
072038			7/16/79	073023	N-O		8/16/79
072997			7/3/79	073078			8/27/79
072122			7/24/79	073077	N-O		8/23/79
072184			8/2/79	073033	N-O		8/16/79
072172			8/2/79	073172			9/11/79
072189	N-O		8/2/79	073213			9/18/79
072188			8/2/79	073075	N-O		8/23/79
072975			6/28/79	073031	N-O		8/16/79
072189			8/2/79	073112	?		?
073142	N-O		9/4/79	073080			8/28/79
073168			9/11/79	073113			8/30/79
072041			7/10/79	072213	N-O		8/7/79
072045	N-O		7/10/79	072056			7/12/79
072186			8/2/79	073217			9/18/79
072039			7/10/79	072019			7/5/79
072042			7/10/79	072988			7/3/79
072980	N-O		6/28/79	072056			7/12/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073212		Mich	9/18/79	072321		Mich.	3/15/79
073245	N-O		9/20/79	072373			3/27/79
072032			7/10/79	072229			8/9/79
072986			7/3/79	072206	N-O		8/7/79
072992	N-O		7/3/79	072174			8/2/79
072212	N-O		8/7/79	072259			3/7/79
072212	N-O		8/7/79	072256			3/7/79
072522			4/17/79	072482			4/10/79
073111			8/30/79	072399	N-O		3/29/79
072057			7/12/79	072202			8/7/79
072029			7/10/79	072228			8/9/79
072491	N-O		4/10/79	072081			7/17/79
072374			?	072203			8/7/79
072481	NO		4/10/79	072205			8/7/79
072239			8/14/79	072104	N-O		7/19/79
072014	NO		7/5/79	072227			8/9/79
073188	NO		9/13/79	072233	N-O		8/9/79
073201			9/13/79	072207	N-O		8/7/79
072100			7/19/79	072101			7/19/79
072288			3/13/79	072202			8/7/79
073268	N-O		9/25/79	072228			8/9/79
072013			7/5/79	072229			8/9/79
072416			3/29/79	072230	N-O		8/9/79
072178			8/2/79	072104	N-O		7/19/79
072541			4/17/79	072522			4/17/79
072230	N-O		8/9/79	072232			8/9/79
072231			8/9/79	072207	N-O		8/7/79
072371	N-O		3/ /79	072254			3/7/79
072399	N-O		3/29/79	072205			8/7/79
072545			4/19/79	072231			8/9/79
072376			3/27/79	072289	N-O		3/13/79
072523			4/17/79	072523			4/17/79
072255			3/7/79	072486			4/10/79
072549			4/19/79	072206	N-O		8/7/79
072524	N-O		4/17/79	072256			3/7/79
072259			3/7/79	072524	N-O	↓	4/17/79
072077	N-O	↓	7/17/79	072490			4/10/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073085		Mich	8/28/79	072079		Mich	7/17/79
073182			9/11/79	072249			8/14/79
073132			9/4/79	072144			7/26/79
073049			8/21/79	073235			9/20/79
073177			9/11/79	073027			8/16/79
073227			9/20/79	073129			9/4/79
073054	N-O		8/21/79	073001			8/14/79
073055	N-O		8/21/79	073023	N-O		8/16/79
073055	N-O		8/21/79	072250			8/14/79
073227			9/20/79	072248			8/14/79
073050			8/21/79	073236			9/20/79
073089			8/28/79	072160			7/30/79
073085			8/28/79	072178			8/2/79
073054	N-O		8/21/79	072218			8/9/79
073092	N-O		8/28/79	072043	N-O		7/10/79
073049			8/21/79	073130			9/4/79
073132			9/4/79	072087			7/19/79
073137	N-O		9/4/79	073326			10/4/79
072971	N-O		6/28/79	072946			6/26/79
072140			7/26/79	072172			8/2/79
072101			7/19/79	072083	N-O		2/17/79
072970			6/28/79	072043	N-O		7/10/79
072105	N-O		7/19/79	072037	N-O		7/14/79
072078			7/17/79	072158			7/31/79
072144			7/26/79	072041			7/10/79
072105	N-O		7/19/79	072175	N-O		8/2/79
072141	N-O		7/26/79	072087			7/17/79
072980	N-O		6/28/79	072225			8/9/79
072140			7/26/79	072042			7/10/79
072204			8/7/79	072157			7/31/79
072142			7/26/79	072084	N-O		7/17/79
072100			7/19/79	072040			7/10/79
072142			7/26/79	073282			9/27/79
073135			9/4/79	072180			8/2/79
072136			7/26/79	072039			7/10/79
072102			7/19/79	073011	N-O		8/14/79
073169	N-O		9/4/79	072037	N-O		7/10/79

(A2) HUMAN CRYOPCOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073141		Mich	8/4/79	072233	N-O	Mich	8/9/79
073009	N-O		8/14/79	07 3043			8/21/79
073003			8/14/79	072826			5/31/79
072782			5/24/79	072227			8/9/79
073175	N-O		9/11/79	072796			5/24/79
073167			9/11/79	072796			5/24/79
073173			9/11/79	072286			8/9/79
072765			5/17/79	072016			7/5/79
072541			4/17/79	073270	N-O		9/25/79
073279			4/27/79	072797	N-O		5/24/79
073266			9/25/79	072792			5/24/79
073286			9/27/79	072789	N-O		5/24/79
072785	N-O		5/24/79	073305	N-O		10/2/79
073287			9/27/79	072794			5/24/79
073146	N-O		9/6/79	072792			5/24/79
073303			10/2/79	073280			8/27/79
073284			9/27/79	073303			10/2/79
073284			9/27/79	072766	N-O		5/22/79
073007	N-O		8/14/79	073267			9/25/79
073306			10/2/79	073305	N-O		10/2/79
072763			5/22/79	073308			10/2/79
072782			5/24/79	073281			4/27/79
073330			10/4/79	072764	N-O		5/22/79
072765			5/22/79	072766	N-O		5/22/79
072779			5/22/79	072792	N-O		5/24/79
073286			4/27/79	073270	N-O		9/25/79
073011	N-O		8/14/79	072794			5/24/79
072786			5/24/79	073308			10/2/79
072773			5/24/79	072001	N-O		7/3/79
072773			5/22/79	072789	N-O		5/24/79
073266			3/25/79	072234	N-O		8/9/79
073206			9/18/79	072209			8/7/79
072777	N-O		5/22/79	072841			5/31/79
072833	N-O		5/31/79	072810	N-O		5/29/79
073047	N-O		8/21/79	072795	N-O		7/3/79
072174			8/2/79	072859	N-O		6/26/79
072764	N-O		5/22/79	072826			5/31/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072867		Mich	6/6/79				
07 2868			6/6/79				
07 2896			6/12/79				
07 2964			6/28/79				
07 2961	N-O		6/26/79				
072165			7/31/79				
07 2167	N-O		7/31/79				
07 2957			6/28/79				
072165			7/31/79				
07 2170	N-O		7/31/79				
07 2867			6/6/79				
07 2961			6/26/79				
07 2166			7/31/79				
07 2170	N-O		7/31/79				
072008			7/5/79				
07 2952			6/26/79				
07 2061	N-O		7/12/79				
07 2006			7/5/79				
07 2006			7/5/79				
072003	N-O		7/3/79				
072007			7/5/79				
072897	N-O		6/12/79				
07 2004			7/3/79				
07 2955			6/26/79				
07 2164			7/31/79				
07 2167	N-O		7/31/79				
07 2959	N-O		6/26/79				
07 2168	N-O		7/31/79				
07 2957			6/24/79				
07 2168	N-O		7/31/79				
07 2962			6/28/79				

HUMAN GAMMA GLOBULIN PRODUCTION CHECKLIST

LOT # IVBG-1B

Note: a) Letters and numbers in parentheses at left refer to steps in Protocol F3.

b) Place date and your initials in underlined space to right when procedure completed.

c) P = protein concentration in mg/ml; TP = total protein, in grams.

(A2) HUMAN CRYOPOOR PLASMA:

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072490	Non-O	Mich	9/5/79	72[illegible]	O	Mich	9/[illegible]
073181	O		9/[illegible]	72[illegible]2	O		9/25/79
072395	O		9/27/79	73156	O		9/6/79
073262	O		9/25/79	72515	A-O		4/12/79
072014	Non-O		9/5/79	72475	O		4/10/79
072095	O		9/19/79	72516	O		4/12/79
072513	O		4/12/79	72472	O		4/5/79
73157	O		9/6/79	72470	A-O		4/5/79
73060	O		9/23/79	72487	O		4/10/79
73161	A-O		9/6/79	72468	O		4/5/79
73200	A-O		9/13/79	72472	O		4/5/79
73260	A-O		9/25/79	72469	O		4/5/79
73092	AO		9/28/79	72325	O		3/27/79
73162	O		9/6/79	72476	O		4/10/79
73158	O		9/6/79	72476	O		4/10/79
73261	O		9/25/79	72477	A-O		4/10/79
73261	O		9/25/79	72478	O		4/10/79
73157	O		9/6/79	72473	O		4/10/79
73154	O		9/6/79	72549	O		4/19/79
73099	O		9/28/79	72482	O		4/10/79
73221	O		9/18/79	72475	O		4/10/79
73260	A-O		9/25/79	72553	O		4/19/79
72833	A-O		5/31/79	72330	O		3/20/79
73149	O		9/6/79	72551	A-O		4/19/79
72868	O		10/10/79	72330	O		3/20/79
72833	A-O		5/31/79	72332	O		3/20/79
73149	O		9/6/79	72478	O		4/10/79
72868	O		10/10/79	72548	O		4/19/79
73195	O		9/13/79	72226	A-O		3/20/79
72194	A-O	V	9/13/79	72093	O	V	7/19/79

MDJ 4/27

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
72098	O	Mich	7/19/79	72232	O	Mich	8/9/79
72780	O		5/23/79	72017	A-O		7/5/79
73206	O		9/18/79	73039	O		8/21/79
72192	O		8/2/79	72019	O		7/5/79
73280	O		9/27/79	73042	A-O		8/21/79
72030	O		7/10/79	72846	O		6/1/79
72204	O		9/13/79	73042	A-O		8/21/79
73272	O		9/27/79	72230	A-O		8/9/79
73271	O		9/27/79	72172	O		5/2/79
73001	O		9/5/79	73043	O		8/21/79
72192	O		8/7/79	73039	O		8/21/79
72098	O		7/19/79	72382	O		3/27/79
72539 ~~72537~~	O		4/12/79	72455	O		4/15/79
72099	A-O		7-19 79	73041	A-O		8/21/79
73279	O		9/27/79	72440	O		4/3/79
72097	O		7/19/79	72450	O		4/5/79
72089	O		7/19/79	72507	O		4/12/79
72099	A-O		7/19/79	72490	O		4/10/79
72092	O		7/19/79	72436	A-O		4/3/79
72091	O		7/19/79	72487	O		4/10/79
72096	O		7/19/79	72434	O		4/3/79
72094	A-O		7/19/79	72504	A-O		4/12/79
72097	O		7/19/79	72433	O		4/3/79
72093	O		7/19/79	72469	O		4/5/79
72095	O		7/19/79	72511	O		4/12/79
72215	A-O		8/7/79	72511	O		4/12/79
72071	O		7/17/79	72504	A-O		4/12/79
72073	O		7/10/79	72571	O		4/24/79
72214	O		8/7/79	72507	O		4/12/79
72215	A-O		8/7/79	72454	A-O		4/5/79
72096	O		7/19/79	72491	A-O		4/10/79
72102	O		7/19/79	72433	O		4/3/79
72226	O		8/9/79	72410	O		3/27/79
73040	O		8/21/79	72494	O		4/10/79
72177	O		8/2/79	72489	O		4/10/79
72161	O		7/3/79	72410	O		3/27/79
72215	O	V	8/20/79	72486	O	V	4/10/79

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073159	O	MICHIGAN	9/6/79	072001	non-O	MICHIGAN	7/3/79
073155	O		9/25/79	073090	non-O		6/22/79
073090	O		9/25/79	073093	O		6/27/79
073156	O		9/6/79	073190	O		9/13/79
073223	non-O		9/18/79	072221	O		9/9/79
073135	O		9/4/79	072241	non-O		6/14/79
073163	non-O		9/6/79	072956	O		6/26/79
073127	O				O		
073161	non-O		9/6/79	072161	O		7/31/79
073163	non-O		9/6/79	073669	non-O		6/23/79
073061	O		9/23/79	072149	non-O		9/26/79
073096	O		9/25/79	072290	O		6/14/79
073263	O		9/25/79	072162	non-O		7/31/79
073200	non-O		9/13/79	072952	O		6/26/79
073050	O		9/23/79	073223	non-O		9/18/79
073336	non-O		10/4/79	072950	O		6/26/79
073160	O		9/23/79	072929	O		5/3/79
073291	O		9/24/79	072011	O		7/5/79
073254	O		9/25/79	072941	O		5/31/79
073255	O		9/25/79	072061	non-O		6/12/79
073050	O		9/21/79	072949	O		6/26/79
073196	O		9/13/79	072955	O		6/26/79
073163	non-O		9/23/79	072011	O		7/5/79
073163	non-O		9/13/79	073095	non-O		6/29/79
073259	O		9/25/79	072162	non-O		6/7/79
073226	non-O		9/18/79	072995	non-O		7/3/79
073061	—		—	072157	O		7/3/79
073296	O		10/2/79	073107	O		6/30/79
073263	O		9/25/79	072240	O		6/14/79
072027	O		5/31/79	072150	O		7/31/79
072063	non-O		7/11/79	072235	non-O		5/9/79
072276	non-O		7/9/79	072152	O		7/3/79
072948	O		6/26/79	072241	non-O		6/14/79
072263	O		9/4/79	072242	O		6/14/79
072949	O		6/26/79	072236	non-O		5/9/79
072959	non-O	MICHIGAN	6/26/79	072149	non-O	MICHIGAN	7/26/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Appendix C2

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
072.12	O	MICHIGAN	9/14/74	72130	non O	mich	7/26/79
[illegible]	[illegible]		[illegible]	72134	non O		7/26/79
[illegible]	[illegible]		[illegible]	72059	O		7/12/79
[illegible]	O		7/31/79	72951	non O		6/26/79
[illegible]	[illegible]		9/13/79	72954	non O		6/26/79
[illegible]	[illegible]		7/31/79	72134	non O		7/26/79
[illegible]	[illegible]		[illegible]	072282	O		7/13/79
[illegible]	[illegible]		9/14/74	072744	non-O		5/17/79
[illegible]	[illegible]		[illegible]	072742	O		5/17/79
[illegible]	O		3/27/79	072744	non-O		5/17/79
[illegible]	[illegible]		9/13/79	072750	O		5/17/79
[illegible]	O		9/13/79	72303	O		3/17/79
72350	non-O		3/27/79	72736	O		5/17/79
[illegible]	O		3/27/79	72737	O		5/17/79
72468	O		4/5/79	72745	non-O		5/17/79
72295	O		3/13/79	72296	O		5/13/79
72297	O		3/13/79	72503	O		4/12/79
72745	non-O		5/17/79	72283	non-O		3/13/79
72301	O		3/13/79	72295	O		3/13/79
72280			3/13/79	72286	non-O		3/13/79
72290	O		3/13/79	72752	O		5/17/79
72683	O		5/8/79	72284	O		3/13/79
72286	non-O		3/13/79	72287	O		3/27/79
72737	O		5/17/79	72750	O		5/17/79
72003	non-O		7/3/79	72289	non-O		3/13/79
72193	O		7/26/79	72281	O		3/13/79
72056	O		7/12/79	72752	O		5/17/79
73040	O		8/21/79	73327	O		10/4/79
72051	non-O		6/26/79	73016	non O		8/16/79
72058	O		7/12/79	73013	O		8/16/79
72133	O		7/26/79	73330	O		10/4/79
72002	O		7/3/79	73013	O		8/16/79
72002	non O		7/12/79	73311	O		6/12/79
72950	O		6/26/79	73019	O		8/16/79
72008	O		7/5/79	73273	non-O		8/27/79
72003	non O		7/12/79	73272	O		8/27/79
72953	O		6/26/79	73268	non-O		9/25/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
73020	O	Mich.	8/16/79	072400	O	MICHIGAN	3/29/79
73020	O		8/16/79	072334	O		3/26/79
73311	O		10/2/79	072329	O		3/26/79
73004	O		8/14/79	072399	O		3/24/79
73204	O		9/13/79	072324	O		3/20/79
73273	non-O		9/27/79	072402	non-O		3/29/79
73267	O		9/25/79	072403	O		3/29/79
73314	non-O		10/2/79	072329	O		3/26/79
73290	non-O		9/27/79	072557	O		4/9/79
73193	O		9/13/79	072532	O		4/17/79
73194	non-O		9/15/79	072431	O		4/3/79
73289	O		9/27/79	072509	O		4/12/79
73288	O		9/27/79	072562	O		4/19/79
73288	O		9/27/79	072704	O		3/13/79
73259	O		4/25/79	072298	O		3/13/79
73019	O		8/16/79	072396	O		3/20/79
73016	non-O		8/16/79	072550	O		4/19/79
73221	O		9/18/79	072752	--		3/7/79
73014	O		3/16/79	072514	O		4/17/79
73335	O		10/4/79	072555	O		4/19/79
73004	O		8/14/79	072396	O		3/29/79
73327	O		10/4/79	072338	O		3/20/79
73014	O		8/16/79	072115	non-O		7/24/79
73335	O		10/4/79	072115	non-O		7/24/79
072494	O		4/10/79	072024	non-O		7/5/79
072760	—		3/7/79	072110	O		7/24/79
072530	O		4/17/79	072021	O		7/5/79
072475	O		4/3/79	072219	O		8/9/79
072262	—		3/7/79	072016	non-O		7/5/79
072260	—		3/7/79	072026	O		7/5/79
072477	non-O		4/10/79	072024	non-O		7/5/79
072406	O		3/29/79	072116	O		7/24/79
072349	O		3/22/79	072017	non-O		7/5/79
072350	O		3/21/79	072022	O		7/5/79
072331	O		3/20/79	072020	O		7/5/79
072331	O	↓	3/20/79	072020	O	↓	7/5/79
072400	O	MICHIGAN	3/29/79	072027	non-O	MICHIGAN	7/5/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
72428	O	Mich	4/3/79	72394	O	Mich	3/27/79
72362	—		3/7/79	72617	O		4/26/79
72424	non O		—	72567	O		4/24/79
72365	O		3/21/79	72431	O		4/3/79
72434	O		4/3/79	72532	O		4/17/79
72440	O		4/3/79	72564	non O		4/19/79
72553	non O		4/5/79	72530	O		4/17/79
72559	O		4/12/79	72470	O		3/29/79
72371	O		3/27/79	72609	—		3/27/79
72379	O		3/27/79	72620	O		4/26/79
72502	O		4/12/79	72595	non O		4/26/79
72329	O		3/27/79	72323	O		3/15/79
72560	O		4/19/79	72604	non O		4/26/79
72558	O		4/19/79	72602	O		4/26/79
72500	O		4/12/79	72598	O		4/26/79
72562	O		4/19/79	72319	O		3/15/79
72428	O		4/3/79	72569	non O		4/24/79
72555	O		4/19/79	72605	O		4/26/79
72571	O		4/24/79	72540	non O		4/17/79
72537	O		4/17/79	72574	non O		4/24/79
72252	—		3/7/79	72569	non O		4/24/79
72548	O		4/19/79	72307	non O		3/15/79
72087	—		7/1/79	72594	O		4/24/79
72501	non O		4/12/79	72342	O		3/20/79
72395	O		3/27/79	72572	O		4/24/79
72333	O		3/20/79	72573	O		4/24/79
72253	—		3/7/79	72574	non O		4/24/79
72565	O		4/19/79	72603	O		4/26/79
72408	O		3/29/79	72594	O		4/24/79
72326	non O		3/20/79	72597	O		4/26/79
72481	non O		4/10/79	72572	O		4/24/79
72551	non O		4/19/79	72319	O		3/15/79
72261	—		3/7/79	72567	O		4/17/79
72355	—		3/7/79	72608	O		4/26/79
72561	non O		4/19/79	72573	O		4/24/79
72261	—		3/7/79	72604	non O		4/26/79
72500	O	↓	4/12/79	72595	non O	↓	4/26/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd): Appendix C2

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
72577	O	Mich	4/26/79	72466	O	Mich	4/5/79
[illegible]	O		[illegible]	72547	O		[illegible]
72306	—		3/15/79	72540	non O		4/17/79
72314	non O		3/15/79	72275	—		3/8/79
72547	O		4/19/79	72545	O		[illegible]
72742	O		5/17/79	72318	O		3/15/79
72416	O		4/3/79	72542	O		4/17/79
72448	O		4/5/79	72305	non O		3/20/79
72443	O		4/3/79	72318	O		3/15/79
72443	O		4/3/79	72459	O		4/5/79
72326	O		3/20/79	72250	—		3/13/79
72335	O		3/20/79	72276	—		3/8/79
72326	O		3/20/79	72463	O		4/5/79
72741	non O		5/17/79	72459	O		4/5/79
72441	O		4/3/79	72415	non O		3/29/79
72314	non O		3/15/79	72415	non O		3/29/79
72340	O		3/20/79	72605	O		4/26/79
72448	O		4/5/79	72273	—		3/8/79
72741	non O		5/17/79	72269	—		3/8/79
72338	O		3/20/79	72542	O		4/17/79
72340	O		3/20/79	72282	O		3/13/79
72441	O		4/3/79	72271	—		3/8/79
72313	non O		3/15/79	72253	—		3/7/79
72306	—		3/15/79	72277	—		3/8/79
72315	O		3/15/79	72407	O		3/29/79
72342	O		3/20/79	72323	O		3/15/79
72446	non O		4/3/79	72684	O		5/8/79
72413	O		3/29/79	72686	O		5/8/79
72309	O		3/15/79	72689	O		5/8/79
72307	non O		3/15/79	72681	O		5/8/79
72407	O		3/28/79	72617	O		4/26/79
72308	non O		3/15/79	72685	non O		5/8/79
72410	O		3/29/79	72684	O		5/8/79
72413	O		3/29/79	72685	non O		5/8/79
72414	O		3/29/79	72635	O		5/1/79
72408	O		3/29/79	72686	O		5/8/79
72463	O	↓	4/5/79	72631	O	↓	5/1/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Appendix C2

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
72689	O	Mich	5/8/79	73171	non O	Mich	9/11/79
72635	O		5/1/79	73167	O		9/11/79
72683	O		5/8/79	72109	non O		7/24/79
72553	O		4/19/79	73143	non O		9/6/79
72631	O		5/1/79	72110	O		7/24/79
72392	non O		3/27/79	72146	non O		7/26/79
72515	O		4/12/79	72177	O		8/2/79
72394	O		3/27/79	73169	non O		9/11/79
72396	O		3/28/79	73145	non O		9/6/79
72501	non O		4/12/79	73170	O		9/11/79
72564	non O		4/19/79	72113	O		7/24/79
72561	non O		4/19/79	72932	O		6/14/79
72392	non O		3/27/79	72976	non O		6/28/79
72503	O		4/12/79	73142	non O		9/4/79
72502	O		4/12/79	72116	O		7/24/79
72845	O		6/1/79	73138	O		9/4/79
72560	O		4/19/79	72114	O		7/24/79
72630	non O		5/1/79	73173	O		9/11/79
72630	non O		5/1/79	72884	O		6/7/79
72385	O		3/27/79	72158	O		7/31/79
72845	O		6/1/79	73170	O		9/11/79
72906	O		6/12/79	73009	non O		8/14/79
72822	O		5/30/79	72857	non O		6/5/79
72185	O		8/2/79	72160	O		7/31/79
72981	O		6/28/79	72219	O		8/9/79
72824	O		5/30/79	72804	O		5/29/79
72821	O		5/29/79	73317	O		10/4/79
72930	O		6/14/79	73328	non O		10/4/79
72824	O		5/30/79	73317	O		10/4/79
72906	O		6/12/79	73145	O		9/6/79
72173	O		8/2/79	72157	non O		7/31/79
72114	O		7/24/79	73003	O		8/14/79
72112	O		7/24/79	72162	non O		7/31/79
72112	O		7/24/79	73314	non O		10/2/79
72208	—		—	73328	non O		10/4/79
72117	O		7/24/79	72224	O		8/9/79
72979	O	V	6/28/79	73007	non O	V	8/14/79

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Appendix C2

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
73146	non O	Mich	9/6/79	072362	non-O	MICHIGAN	3/21/79
[illegible]	O	"	5/29/79	07231-4	O		3/21/79
[illegible]	non O	"	8/16/79	072367	"		3/21/79
72870	O		6/6/79	072369	O		3/21/79
73031	non O		8/16/79	072362	non-O		3/21/79
72211	O		8/7/79	072365	O		3/21/79
72880	non O		6/7/79	072352	O		3/22/79
72870	O		6/6/79	072962	O		6/29/79
72857	non O		6/5/79	072941	O		6/26/79
72884	O		6/7/79	072119	O		7/24/79
72211	O		8/7/79	072939	O		6/26/79
72860	O		6/5/79	072123	non-O		7/24/79
72872	O		6/7/79	073169	O		9/11/79
72210	O		8/7/79	072407	non-O		—
72803	O		5/29/79	072939	non-O		6/26/79
72887	O		6/7/79	072118	O		7/24/79
72814	O		5/29/79	072040	O		9/10/79
072363	O		3/27/79	072944	O		6/26/79
072378	O		3/27/79	072945	non-O		6/26/79
072423	O		—	072047	non-O		7/16/79
072528	non-O		4/17/79	072045	non-O		9/10/79
072373	O		3/27/79	072120	O		7/24/79
072352	O		—	072438	O		6/26/79
072528	non-O		4/13/79	072880	non-O		6/7/79
072351	O		3/27/79	072906	O		5/29/79
072349	O		3/27/79	078016	O		7/5/79
072397	O		3/20/79	072914	O		5/29/79
072376	O		3/27/79	072172	O		7/24/79
072410	O		3/24/79	072944	non-O		6/26/79
072368	O		3/21/79	072910	non-O		5/29/79
072363	O		3/21/79	072960	O		6/5/79
072525	O		—	072119	O		7/24/79
072350	O		3/21/79	072907	O		6/7/79
072346	non-O		3/20/79	072209	O		6/7/79
072371	non-O		3/27/79	072960	O		6/26/79
072346	non-O		3/20/79				
072347	O		3/20/79				

(A2) HUMAN CRYOPOOR PLASMA (cont'd): Appendix C2

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073097	min-O	MICHIGAN	8/28/79				
073175	min-O		9/11/79				
072094	min-O		7/19/79				
072195	O		8/7/79				
072209	O		8/7/79				
073114	O		8/30/79				
072021	O		7/5/79				
073163	min-O		9/11/79				
072026	O		9/5/79				
072019	min-O		7/5/79				
072095	min-O		5/24/79				
072022	O		7/5/79				
072027	min-O		7/5/79				
072074	O		5/22/79				
072063	O		5/22/79				
072090	O		5/23/79				
072096	O		5/24/79				
073300	O		10/2/79				
071775	O		5/22/79				
071775	O		5/21/79				
072210	O		8/7/79				
072425	O		4/3/79				
072374	O		—				
072525	O		—				
072398	O		3/27/79				
72941	O		6/26/79				
72959	min-O		6/26/79				
72945	min-O		[illegible]				
23145	O		9/6/79				
72126	min-O		7/24/79				
72125	O		7/24/79				
72892	O		[illegible]				

HUMAN GAMMA GLOBULIN PRODUCTION CHECKLIST

LOT # 1VBG-2-A

Notes: a) Letters and numbers in parentheses at left refer to steps in Protocol F3.

b) Place date and your initials in underlined space to right when procedure completed.

c) P = protein concentration in mg/ml; TP = total protein, in grams.

(A2) HUMAN CRYOPOOR PLASMA:

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153643	O	FT DETK.	9/30/80	153668	non-O	FT Dick	10/2/80
153639	O		9/30/80	153668	non-O		10/2/80
153643	O		9/30/80	153663	O		10/2/80
153646	non-O		9/30/80	153806	non-O		10/21/80
153646	non-O		9/30/80	073770	O		6/5/80
153649	O		9/30/80	073771	O		6/5/80
153640	O		9/30/80	073795	non-O		6/10/80
153637	O		9/30/80	073780	O		6/5/80
153637	O		9/30/80	073772	O		6/5/80
153638	non-O		9/30/80	073779	O		6/5/80
153640	O		9/30/80	073771	O		6/5/80
153638	non-O		9/30/80	073789	O		6/10/80
153650	O		10/2/80	073789	O		6/10/80
153659	non-O		10/2/80	073772	O		6/5/80
153635	O		9/30/80	073774	O		6/10/80
153639	O		9/30/80	073783	O		6/10/80
153647	non-O		9/30/80	073788	O		6/10/80
153847	O		10/28/80	073779	O		6/5/80
153844	O		10/28/80	073770	O		6/5/80
153844	O		10/23/80	073772	O		1/29/80
153674	non-O		10/2/80	073380	O		1/31/80
153665	O		10/2/80	073782	non-O		6/5/80
153667	O		10/2/80	073786	non-O		6/10/80
153653	O		10/2/80	073783	O		6/10/80
153659	non-O		10/2/80	073782	non-O		6/5/80
153814	non-O		10/21/80	073400	O		2/7/80
153847	O		10/28/80	073402	non-O		2·7·80
153818	non-O		10/23/80	073405	O		2·7·80
153661	O		10/2/80	073402	non-O		2·7·80
153661	O	↓	10/2/80	073405	O	↓	2·7·80

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153220	O	Ft Det	8/7/80	073885	O	Ft Detr	6/19/80
153218	non-O		8/7/80	073864	O		6/19/80
153215	O		8/7/80	073881	O		6/19/80
153220	O		8/7/80	073865	non-O		6/19/80
073860	O		8/7/80	073874	O		6/19/80
073882	O		6/19/80	073860	O		6/19/80
073869	O		6/19/80	073892	O		6/19/80
073876	O		6/19/80	073876	O		6/19/80
073874	O		6/19/80	073872	non-O		6/19/80
073886	non-O		6/19/80	073395	non-O		2/5/80
073863	non-O		6/19/80	073396	O		2/5/80
073863	non-O		6/19/80	073397	O		2/5/80
073707	O		6/16/80	073392	O		2/5/80
073707	O		6/16/80	073397	O		2/5/80
073709	non-O		6/16/80	073394	non-O		2/5/80
073413	O		2/12/80	153345	O		8/21/80
073413	O		2/12/80	153350	O		8/26/80
073399	O		2/7/80	153258	O		8/12/80
073399	O		2/7/80	153287	non-O		8/14/80
073794	O		6/16/80	153300	O		8/19/80
073400	O		2/7/80	153260	O		8/12/80
073780	O		6/5/80	153262	O		8/12/80
073795	non-O		6/16/80	153289	O		8/14/80
073785	O		6/16/80	153341	O		8/21/80
153229	O		8/7/80	153350	O		8/26/80
153229	O		8/7/80	153262	O		8/12/80
153222	O		8/7/80	153284	O		8/14/80
153216	O		8/7/80	153333	O		8/21/80
153219	non-O		8/7/80	153333	O		8/21/80
073851	O		6/19/80	153351	non-O		8/26/80
073806	non-O		6/19/80	153351	non-O		8/26/80
073864	O		6/19/80	153284	O		8/14/80
073865	non-O		6/19/80	153346	O		8/26/80
073869	O		6/19/80	153345	O		8/21/80
073859	O		6/19/80	153322	O		8/21/80
073872	non-O	↓	6/19/80	153322	O		8/21/80
073885	O	Ft Det	6/19/80	153346	O	↓	8/26/80

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153325	non-O	FH	8/21/80	15350'	O	FH	9/11/80
153324	O		8/21/80	153483	O		9/11/80
153325	non-O		8/21/80	153490	non-O		9/11/80
153335	O		8/21/80	153463	O		9/11/80
153311	O		8/19/80	153493	O		9/11/80
153324	O		8/21/80	073888	O		6/19/80
153311	O		8/19/80	073871	O		6/19/80
153111	non-O		7/24/80	153503	non-O		9/11/80
153264	O		8/12/80	153498	O		9/11/80
153246	non-O		8/12/80	073892	O		6/24/80
153258	O		8/12/80	153506	non-O		9/11/80
153264	O		8/12/80	153050	O		7/15/80
153246	non-O		8/12/80	153073	O		7/17/80
153265	O		8/12/80	153052	O		7/15/80
153300	O		8/19/80	153067	O		7/17/80
152287	non-O		8/14/80	153518	non-O		9/16/80
153291	O		8/14/80	153084	O		7/22/80
153289	O		8/14/80	153062	non-O		7/17/80
153291	O		8/14/80	153064	non-O		7/17/80
153240	O		8/12/80	153079	O		7/17/80
153503	non-O		9/11/80	153532	non-O		9/16/80
153500	non-O		9/11/80	153072	O		7/17/80
153260	O		8/12/80	153070	O		7/17/80
153242	O		8/12/80	153060	O		7/17/80
153265	O		8/12/80	153614	O		9/16/80
153493	O		9/11/80	153508	O		9/11/80
153490	non-O		9/11/80	153514	O		9/16/80
153498	O		9/11/80	153531	non-O		9/16/80
153494	O		9/11/80	153528	O		9/16/80
153497	non-O		9/11/80	153499	O		9/11/80
153501	O		9/11/80	153508	O		9/11/80
153481	O		9/9/80	153522	O		9/16/80
153500	non-O		9/11/80	153522	O		9/16/80
153494	O		9/11/80	153512	O		9/16/80
153481	O		9/9/80	153517	O		9/16/80
153496	O		9/11/80	153067	O		7/17/80
153497	non-O		9/11/80	153524	O		9/16/80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073396	O	Ft. Detr	2.5.80	153045	non-O	Ft. Det	7.15.80
073381	O		1.31.80	153102	O		7.22.80
073381	O		1.31.80	153102	O		7.22.80
073407	non-O		2.7.80	153078	non-O		7.17.80
073395	non-O		2.5.80	153079	non-O		7.17.80
073402	non-O		2.7.80	153097	O		7.22.80
073394	non-O		2.5.80	153090	O		7.22.80
073392	O		2.5.80	153090	O		7.22.80
153272	non-O		8.15.80	153105	O		7.22.80
153299	non-O		8.19.80	153105	O		7.22.80
153296	O		8.19.80	153095	non-O		7.22.80
153296	O		8.19.80	153106	non-O		7.22.80
153307	O		8.19.80	153106	non-O		7.22.80
153307	O		8.19.80	153109	O		7.24.80
153298	O		8.19.80	153098	non-O		7.22.80
153308	O		8.19.80	153111	non-O		7.24.80
153272	non-O		8.15.80	153100	O		7.22.80
153268	non-O		8.12.80	153100	O		7.22.80
153269	non-O		8.12.80	153109	O		7.24.80
153280	O		8.14.80	153101	O		7.22.80
153280	O		8.14.80	153112	O		7.24.80
153281	O		8.14.80	153101	O		7.22.80
153281	O		8.14.80	153097	O		7.22.80
153299	non-O		8.19.80	153112	O		7.24.80
153339	O		8.21.80	153495	O		9/2/80
153341	O		8.21.80	153566	O		9/23/80
153335	O		8.21.80	073695	O		5/27/80
153339	O		8.21.80	073683	non-O		5/27/80
153037	O		7.15.80	153577	non-O		9/23/80
153056	O		7.15.80	153577	non-O		9/23/80
153032	non-O		7.10.80	153582	O		9/23/80
153029	O		7.10.80	153578	O		9/23/80
153045	non-O		7.15.80	073688	non-O		5/27/80
153037	O		7.15.80	153569	non-O		9/23/80
153039	non-O		7.15.80	153582	O		9/23/80
153032	non-O		7.10.80	153569	non-O		9/23/80
153049	O		7.15.80	153586	non-O		9/23/80

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153486	non-O	Ft Detr	9/11/80	073902	O	Ft Detr	6/24/80
153496	O		9/11/80	073911	non-O		6/24/80
153496	non-O		9/11/80	073967	O		6/19/80
073898	O		6/19/80	073908	O		6/24/80
073912	O		6/24/80	073954	O		6/11/80
153506	non-O		9/11/80	073958	O		6/17/80
073912	O		6/24/80	073954	O		6/17/80
073911	non-O		6/24/80	073909	O		6/24/80
073942	O		6/24/80	073999	non-O		6/24/80
073990	O		6/19/80	073991	non-O		6/24/80
073990	O		6/19/80	073991	non-O		6/24/80
073780	non-O		6/3/80	073849	non-O		6/17/80
073878	non-O		6/19/80	073932	O		6/17/80
073977	O		6/19/80	153417	non-O		9.2.80
073903	O		6/24/80	153403	O		9.2.80
073759	O		6/5/80	153403	O		9.2.80
073770	O		6/3/80	073859	O		6.17.80
073754	O		6/5/80	153415	O		9.2.80
073757	O		6/5/80	153420	O		9.2.80
073903	O		6/24/80	153420	O		9.2.80
073878	non-O		6/19/80	153418	O		9.2.80
073742	O		6/3/80	153418	O		9.2.80
073744	non-O		6/3/80	153129	O		7.24.80
073748	non-O		6/3/80	153131	O		7.24.80
073757	O		6/5/80	153122	O		7.24.80
073955	O		6/17/80	153122	O		7.24.80
073909	O		6/24/80	153417	non-O		9.2.80
073909	O		6/24/80	153394	O		8.28.80
073958	O		6/17/80	153412	O		7.2.80
073735	O		6/3/80	153404	non-O		9.2.80
073735	O		6/3/80	153134	O		7.29.80
073744	non-O		6/3/80	153131	O		7.24.80
073956	O		6/17/80	153134	O		7.29.80
073902	O		6/24/80	153412	O		9.2.80
073899	non-O		6/24/80	153401	O		9.2.80
073955	O	"	6/17/80	153398	O		8.28.80
073951	O	Ft Detr	6/17/80	153396	O	"	8.28.80

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153147	O	Ft De	7/29/80	153072	O	Ft De	7/17/80
153584	O		9/23/80	153001	O		7/8/80
153568	O		9/23/80	153084	O		7/22/80
153584	O		9/23/80	153052	O		7/15/80
153156	O		7/29/80	153064	non-O		7/17/80
153558	non-O		9/18/80	153060	O		7/17/80
153578	O		9/23/80	153523	O		9/16/80
153564	O		9/23/80	153518	non-O		9/16/80
153553	O		9/18/80	153534	non-O		9/16/80
153553	O		9/18/80	153528	O		9/16/80
153566	O		9/23/80	073837	non-O		6/17/80
153586	non-O		9/23/80	153522	non-O		9/16/80
153568	O		9/23/80	153782	non-O		10/16/80
153573	O		9/23/80	153787	O		10/21/80
153558	non-O		9/18/80	153778	non-O		10/16/80
153571	non-O		9/23/80	153531	non-O		9/16/80
073842	O		6/17/80	153791	O		10/21/80
073842	O		6/17/80	153801	O		10/21/80
073886	O		6/16/80	153784	O		10/16/80
073850	O		6/17/80	153804	O		10/21/80
073867	O		6/19/80	153525	non-O		9/16/80
073832	O		6/12/80	153525	non-O		9/16/80
073856	O		6/17/80	153524	O		9/16/80
073836	O		6/16/80	153534	non-O		9/16/80
073852	non-O		6/12/80	153499	O		9/11/80
073851	O		6/17/80	153523	O		9/16/80
073852	non-O		6/17/80	154266	non-O		12/18/80
073848	non-O		6/17/80	154267	non-O		12/18/80
153021	O		7/10/80	154214	non-O		12/11/80
153007	O		7/8/80	154215	non-O		12/11/80
153017	O		7/10/80	154242	non-O		12/16/80
153016	non-O		7/10/80	154229	non-O		12/16/80
153031	O		7/10/80	154229	non-O		12/16/80
153073	O		7/14/80	073830	O		6/12/80
153009	O		7/8/80	073831	O		6/12/80
153049	non-O		7/18/80	154239	non-O		12/16/80
153050	O		7/17/80	154270	O		12/18/80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Dono #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153396	O	Ft Det	8.28.80	073389	O	FT.Det	2.5.80
073385	O		1.31.80	153247	O		8.12.80
153394	O		8.28.80	153212	non O		8.5.80
073386	O		2.5.80	153235	O		8.7.80
073396	O		2.5.80	153212	non O		8.5.80
073384	O		1.31.81	153237	O		8.7.80
153395	O		8.28.80	153055	O		7.22.80
073365	O		1.29.80	153050	O		7.17.80
073363	O		1.29.80	153085	O		7.22.80
153401	O		9.2.80	153070	O		7.12.80
153404	non O		9.2.80	153080	O		7.17.80
153376	non O		8.28.80	073353	non O		1.31.80
153376	non O		8.28.80	153004	O		7.8.80
073377	O		1.31.80	153079	O		7.17.80
073388	non O		2.5.80	153062	non O		7.17.80
073384	O		1.31.81	153813	non-O		10/21/80
073380	O		1.31.80	153816	O		10/21/80
073377	O		1.31.80	153815	non-O		10/21/80
073365	O		1.28.80	153818	non-O		10/23/80
073363	O		1.29.80	154242	non O		12.16.80
073383	non O		1.31.80	154239	non O		12.16.80
073382	non O		1.31.81	154269	non O		12.18.80
073389	O		2.5.80	153104	non O		7.22.80
073382	non O		1.31.80	154263	O		12.18.80
073385	O		1.31.80	154237	O		12.16.80
153238	O		8.7.80	154157	non O		12.4.80
153215	O		8.7.80	154159	non O		12.4.80
153237	O		8.7.80	154217	O		12.11.80
153247	O		8.12.80	154270	O		12.18.80
073372	O		1.29.80	154163	O		12.4.80
073388	non O		2.5.80	153564	O		9.23.80
153222	O		8.7.80	154263	O		12.18.80
153232	O		8.7.80	154266	non O		12.15.80
153235	O		8.7.80	153810	O		10.21.80
153216	O		8.7.80	153805	O		10.21.80
153232	O		8.7.80	153813	non O		10.21.80
153232	O		8.7.80	154163	O		12.4.80

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
154270	O	Ft Det	12/18/80	073428	O	Ft Det	2/14/80
154265	non-O		12/18/80	073449	O		2/21/80
154244	O		12/16/80	073696	O		5/27/80
154247	non-O		12/18/80	073419	O		2/12/80
154247	non-O		12/18/80	073371	non-O		1/29/80
154265	non-O		12/18/80	153449	O		9/4/80
073838	non-O		6/12/80	073362	non-O		1/24/80
073838	non-O		6/12/80	073370	non-O		1/23/80
154215	non-O		12/11/80	153849	O		9/4/80
154244	O		12/16/80	073347	O		1/22/80
073697	O		5/27/80	073347	O		1/22/80
073701	O		5/27/80	073349	O		1/22/80
073697	O		5/27/80	073349	non-O		1/22/80
073698	O		5/27/80	153428	O		9/4/80
073689	non-O		5/27/80	153953	O		9/4/80
073690	O		5/27/80	153014	O		7/10/80
073680	O		5/27/80	153438	O		9/4/80
073688	non-O		5/27/80	153436	O		9/4/80
073693	non-O		5/27/80	073349	non-O		1/22/80
073700	O		5/27/80	073352	O		1/24/80
073699	non-O		5/27/80	153429	O		9/4/80
073694	non-O		5/27/80	153444	O		9/4/80
073718	non-O		5/29/80	073996	O		7/9/80
073701	O		5/27/80	153011	O		7/8/80
073693	non-O		5/27/80	073996	O		7/8/80
073700	O		5/27/80	153006	non-O		7/8/80
073710	non-O		5/29/80	153009	O		7/9/80
073438	O		2/19/80	153017	O		7/10/80
073427	O		2/14/80	153014	O		7/10/80
073419	O		2/12/80	153019	non-O		7/10/80
073433	O		2/19/80	153021	O		9/10/80
073696	O		5/27/80	153002	non-O		7/8/80
073450	O		2/21/80	152006	non-O		7/4/80
073450	O		2/21/80	153007	O		7/8/80
073438	O		2/19/80	153016	non-O		7/10/80
073433	O		2/19/80	153002	non-O		7/8/80
073427	O	Ft Det	2/14/80	073449	O	Ft Det	2/21/80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
154163	80	Ft. Det	12.4.80	073716	non O	Ft Det	5.29.80
153816	O		10.21.80	073715	O		5.29.80
153817	O		10.23.80	073715	O		5.29.80
153810	O		10.21.80	073715	O		5.29.80
153805	O		10.21.80	073723	O		5.29.80
153843	non O		10.23.80	073723	O		5.29.80
153814	non O		10.21.80	073720	O		5.29.80
153815	non O		10.21.80	073722	O		5.29.80
153817	O		10.23.80	073726	O		5.29.80
153121	O		7.24.80	073722	O		5.29.80
153116	non O		7.24.80	073715	O		5.29.80
153143	O		10.28.80	073726	O		5.29.80
153848	O		10.28.80	073732	non O		6.3.80
153129	non O		7.24.80	073730	O		5.29.80
153129	O		7.24.80	073360	O		1.24.80
153128	non O		7.24.80	073343	O		1.24.80
153116	non O		7.24.80	073362	non O		1.24.80
153104	non O		7.22.80	073370	non O		1.23.80
153137	non O		7.29.80	153001	O		7.8.80
153138	O		7.29.80	153011	O		7.8.80
153121	O		7.24.80	073356	O		1.24.80
153138	O		7.29.80	073352	O		1.24.80
153127	O		7.24.80	073368	O		1.29.80
153127	O		7.24.80	073356	O		1.24.80
153137	non O		7.29.80	073368	O		1.29.80
153126	O		7.24.80	073360	O		1.24.80
153125	O		7.24.80	153425	O		9.2.80
073645	O		5.27.80	153441	non O		9.4.80
073650	O		5.27.80	073361	non O		1.24.80
153571	non O		9.23.80	073371	non O		1.29.80
153573	O		9.23.80	153726	O		10.9.80
153726	O		10.9.80	073359	non O		1.24.80
073650	O		5.27.80	153435	O		9.4.80
073720	O		5.29.80	153435	O		9.4.80
073732	non O		6.3.80	153425	O		9.2.80
073706	O		5.24.80	153434	non O		9.4.80
073716	non O		5.29.80	153453	O		9.4.80

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
073686	O	Ft Det	5.27.80	073992	O	Ft. Det	7.8.80
153453	O		9.4.80	073976	O		7.3.80
073359	non O		1.24.80	073976	O		2.3.80
153461	non O		9.9.80	073949	non O		7.1.80
153461	non O		9.9.80	073940	O		6.26.80
153441	non O		9.9.80	073988	O		7.8.80
153444	3		9.9.80	073940	O		6.26.80
153446	O		9.4.80	073971	O		7.3.80
073696	O		8.27.80	073945	O		7.1.80
153446	O		9.4.80	~~Pt 000088~~			~~7.3.80~~
153445	O		9.9.80	073989			7.3.80
153434	non O		9.4.80				
153445	O		9.9.80				
073428	O		2.14.80				
153680	non O		10.7.80				
153724	non O		10.9.80				
153679	O		10.7.80				
153705	O		10.7.80				
153681	O		10.7.80				
153724	non O		10.9.80				
153725	non O		10.9.80				
153725	non O		10.9.80				
153694	O		10.7.80				
153681	O		10.7.80				
153~~727~~	O		10.9.80				
153687	O		10.7.80				
153687	O		10.7.80				
153730	O		10.14.80				
073361	non O		1.24.80				
153680	non O		10.7.80				
153727	O		10.9.80				
153679	O		10.7.80				
073931	O		6.2.80				
073931	O		6.26.80				
073988	O		7.8.80				
153004	O		7.8.80				
073992	O		7.8.80				

HUMAN GAMMA GLOBULIN PRODUCTION CHECKLIST

LOT # IVBG-2B

Note: a) Letters and numbers in parentheses at left refer to steps in Protocol F3.

b) Place date and your initials in underlined space to right when procedure completed.

c) P = protein concentration in mg/ml; TP = total protein, in grams.

(A2) HUMAN CRYOPOOR PLASMA:

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
73982	O	FHN	7-3-80	73775	O	FHN	6-5-80
73980	O		7-3-80	73752	O		6-3-80
73977	non-O		7-3-80	153758	O		10-14-80
73948	O		7-1-80	153759	O		10-16-80
73973	non-O		7-3-80	73753	O		6-3-80
73991	non-O		7-8-80	73751	O		6-3-80
73991	non-O		7-8-80	73768	O		6-5-80
73959	O		7-1-80	153747	non-O		10-14-80
73974	O		7-3-80	153747	non-O		10-14-80
73974	O		7-3-80	153715	O		10-9-80
73971	O		7-3-80	153742	O		10-14-80
73973	non-O		7-3-80	153745	O		10-14-80
73959	O		7-1-80	153733	non O		10-14-80
73959	O		7-1-80	153743	non O		10-14-80
73961	non-O		7-1-80	153738	O		10-14-80
153764	non-O		10-16-80	153736	O		10-14-80
153759	O		10-16-80	153736	O		10-14-80
73754	O		6-3-80	153744	O		10-14-80
153764	non-O		10-16-80	154157	non-O		12-4-80
73776	non-O		6-5-80	154237	O		12-16-80
153768	non-O		10-16-80	154175	non O		12-9-80
73754	O		6-3-80	154187	non O		12-9-80
73768	O		6-5-80	154208	non O		12-11-80
73775	O		6-5-80	154217	O		12-11-80
153744	O		10-14-80	154208	non O		12-11-80
153742	O		10-14-80	154187	non O		12-9-80
73753	O		6-3-80	154181	O		12-9-80
73755	non-O		6-3-80	154191	O		12-9-80
153733	non-O		10-14-80	154159	non O		12-4-80
153738	O		10-14-80	154214	non O		12-11-80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
154191	O	[illegible]	12-9-80	73698	O	[illegible]	5-27-80
154206	O		12-11-80	153362	O		8-28-80
154208	O		12-11-80	153365	O		8-26-80
154186	non O		12-4-80	153368	O		8-26-80
153597	O		9-25-80	153382	O		8-28-80
154175	non O		12-9-80	153369	O		8-26-80
154186	non O		12-4-80	153369	O		8-26-80
153590	O		9-23-80	153356	O		8-26-80
153591	non O		9-23-80	153375	O		8-28-80
153593	O		9-25-80	153356	O		8-26-80
154181	O		12-9-80	153383	O		8-28-80
153581	O		9-23-80	153370	non O		8-26-80
153580	O		9-23-80	153369	O		8-26-80
153591	O		9-23-80	153363	O		8-26-80
153715	O		10-9-80	153363	O		8-26-80
153596	non O		9-25-80	153365	O		8-26-80
153768	non O		10-16-80	73746	O		6-3-80
153758	O		10-14-80	73749	O		6-3-80
153590	non O		9-23-80	73857	non O		6-17-80
153589	O		9-23-80	73827	non O		6-12-80
153590	non O		9-23-80	73828	O		6-12-80
73703	O		5-27-80	73746	O		6-3-80
73710	non O		5-29-80	153383	O		8-28-80
153387	non O		8-28-80	173748	non O		6-3-80
153391	O		8-28-80	73745	O		6-3-80
73709	O		5-29-80	73734	O		6-3-80
73694	non O		5-27-80	73745	O		6-3-80
73699	non O		5-27-80	73818	O		6-12-80
153375	O		8-28-80	73818	O		6-12-80
153389	O		8-28-80	153370	non O		8-26-80
73703	O		5-27-80	73749	O		6-3-80
73714	O		5-29-80	73734	O		6-3-80
73714	O		5-23-80	73827	non O		6-12-80
153387	non O		8-28-80	73819	non O		6-12-80
73709	O		5-23-80	73822	O		6-12-80
153391	O		8-28-80	73820	non O		6-12-80
153389	O	"	8-28-80	73740	O	"	6-3-80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
73733	O	FHCRC	6-3-80	153153	O	FHCRC	7-29-80
73733	O		6-3-80	153171	non O		7-31-80
73750	non O		6-3-80	153150	O		7-29-80
73819	non O		6-12-80	153182	O		7-31-80
73824	O		10-12-80	73751	O		6-3-80
73823	O		6-12-80	73767	non O		6-5-80
73825	non O		6-12-80	73760	non O		6-5-80
73830	O		6-12-80	153148	O		7-29-80
73824	O		6-12-80	153154	O		7-29-80
153156	O		7-29-80	73806	O		6-10-80
73774	non O		6-5-80	73803	non O		6-10-80
153150	O		7-29-80	73762	O		6-5-80
153181	O		7-31-80	153167	O		7-31-80
73822	O		6-12-80	153167	O		7-31-80
73828	O		6-12-80	73805	O		6-10-80
73823	O		6-12-80	73807	non O		6-10-80
153152	O		7-29-80	73762	O		6-5-80
153154	O		7-29-80	73796	O		6-10-80
153148	O		7-29-80	153887	non O		10-30-80
153147	O		7-29-80	73797	O		6-10-80
73831	O		6-12-80	73799	O		6-10-80
73825	non O		6-12-80	73801	non O		6-10-80
73820	non O		6-12-80	73804	O		6-10-80
153152	non O		7-29-80	73796	O		6-10-80
153153	O		7-29-80	73807	non O		6-10-80
153181	O		7-31-80	73721	O		5-29-80
153171	non O		731-80	73803	non O		6-10-80
73776	non O		6-5-80	73755	non O		6-3-80
73777	O		6-5-80	73804	O		6-10-80
73760	non O		6-5-80	73801	non O		6-10-80
73774	non O		6-5-80	73805	O		6-10-80
153182	O		7-31-80	73721	O		5-29-80
153183	O		7-31-80	73718	non O		5-29-80
153183	O		7-31-80	153204	O		5-5-80
73767	non O		6-5-80	73719	O		5-29-80
73777	O		6-5-80	73730	O		5-29-80
73752	O	✓	6-3-80	73812	O	✓	6-12-80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
73708	non O	[illegible]	5-24-80	153657	non O	[illegible]	10-2-80
73810	O		6-12-80	153885	O		10-30-80
73813	O		6-12-80	153885	O		—
73811	non O		6-12-80	153884	O		10-30-80
73812	O		6-12-80	153188	O		8-5-80
73706	O		5-29-80	153192	non O		9-5-80
73810	O		6-12-80	153612	O		9-25-80
73708	non O		5-29-80	153878	O		10-30-80
73813	O		6-12-80	153657	non O		10-2-80
73811	non O		6-12-80	153635	O		9-30-80
73806	O		6-10-80	153204	O		8-5-80
153873	O		10-30-80	153607	non O		9-25-80
153559	non O		9-18-80	153608	O		9-25-80
153926	non O		11-4-80	153608	O		9-25-80
153874	non O		10-30-80	153211	O		8-5-80
153626	non O		9-30-80	153633	non O		11-30-80
153927	non O		11-4-80	153622	O		9-30-80
153928	O		11-4-80	153625	O		9-30-80
153888	O		10-30-80	153611	O		9-25-80
153892	non O		10-30-80	153616	non O		9-25-80
153927	non O		11-4-80	153623	O		9-30-80
153924	non O		11-4-80	153618	non O		9-25-80
153926	non O		11-4-80	153627	O		9-30-80
153928	O		11-4-80	153620	O		9-25-80
153878	O		10-30-80	153618	non O		9-25-80
153894	non O		10-30-80	153611	O		9-25-80
153924	non O		11-4-80	153616	non O		9-25-80
153626	non O		9-30-80	153610	O		9-25-80
153889	non O		10-30-80	153623	O		9-30-80
153633	non O		9-30-80	153180	O		7-31-80
153881	non O		10-30-80	153209	O		8-5-80
153649	O		—	153180	O		7-31-80
153980	O		11-11-80	153620	O		9-25-80
153650	O		10-2-80	153610	O		9-25-80
153647	non O		9-30-80	153627	O		9-30-80
153885	O		10-30-80	153622	O		9-30-80
153653	O	↓	10-2-80	153625	O	↓	9-30-80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153837	O	FT Detroit	10/23/80	154099	O	FT Detroit	11/27/80
153754	O		10/14/80	154134	non O		12/2/80
15377	O		10/16/80	073907	O		6/24/80
153823	O		10/23/80	154136	non O		12/2/80
153836	O		10/23/80	073904	non O		6/24/80
153823	O		10/23/80	073906	O		6/24/80
153835	non O		10/23/80	154138	O		12/2/80
153839	non O		10/23/80	154133	non O		12/2/80
153755	non O		10/14/80	154133	non O		12/2/80
153754	O		10/14/80	154131	O		12/2/80
153774	non O		10/16/80	154131	O		12/2/80
153775	O		10/16/80	073918	non O		6/26/80
153775	O		10/16/80	154105	O		11/27/80
153829	O		10/23/80	154095	non O		11/27/80
153835	non O		10/23/80	154105	O		11/27/80
153756	non O		10/14/80	154134	non O		12/2/80
153756	non O		10/14/80	154138	O		12/2/80
153755	non O		10/14/80	154162	non O		12/4/80
153752	O		10/14/80	154107	non O		11/27/80
153833	O		10/23/80	154107	non O		11/27/80
153772	O		10/16/80	154110	non O		11/27/80
153769	O		10/16/80	154111	non O		11/27/80
153753	non O		10/14/80	154110	non O		11/27/80
153771	O		10/16/80	154111	non O		11/27/80
153774	non O		10/16/80	154162	non O		12/4/80
153771	O		10/16/80	154128	non O		12/2/80
153753	non O		10/14/80	154128	non O		12/2/80
153776	O		10/16/80	154095	non O		11/27/80
153757	O		10/14/80	154099	O		11/27/80
153776	O		10/16/80	153915	non O		11/4/80
153769	O		10/16/80	154000	non O		11/13/80
153777	O		10/16/80	153980	O		11/11/80
073899	O		6/24/80	153996	O		11/13/80
073906	O		6/24/80	153716	O		10/9/80
073935	O		6/26/80	153704	non O		10/7/80
073904	non O		6/24/80	153991	O		11/13/80
154136	non O		12/2/80	153997	non O		11/13/80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
15 3783	non-O	[illegible]	10-16-80	154024	non O	[illegible]	11/18/80
15 3803	O		10-21-80	154000	non O		11/13/80
15 3837	O		10-23-80	154027	O		11/18/80
15 3829	O		10-23-80	153876	non O		10/30/80
15 3833	O		10-23-80	154080	non O		11/25/80
15 3834	non-O		10-23-80	154053	non O		11/20/80
15 3838	O		10-23-80	154053	non O		11/20/80
15 3836	O		10-23-80	154031	non O		11/18/80
15 3787	O		10-21-80	154031	non O		11/18/80
153806	non-O		10-21-80	154052	O		11/20/80
15 3785	O		10-16-80	154054	non O		11/20/80
153 782	non O		10-16-80	153858	O		10/28/80
15 3785	O		10-16-80	153870	non O		10/28/80
15 3784	O		10-16-80	153854	non O		10/28/80
15 3789	non-O		10-21-80	153862	non O		10/28/80
153789	non-O		10-21-80	153866	non O		10/18/80
15 3589	O		9-23-80	153864	O		10/28/80
15 378 3	non-O		10-16-80	153873	O		10/30/80
153803	O		10-21-80	153854	non O		10/18/80
15 3792	non-O		10-21-80	153603	non O		9/25/80
15 3778	non-O		10-16-80	153599	O		9/25/80
15 380 4	O		10-21-80	153614	non O		9/25/80
15 3790	non O		10-21-80	153886	non O		10/28/80
15 3791	O		10-21-80	153876	non O		10/30/80
153976	non O		11/11/80	153870	non O		10/28/80
154005	O		11/13/80	153842	non O		10/23/80
153973	O		11/11/80	153843	non O		10/23/80
154009	non O		11/18/80	153860	non O		10/28/80
153921	O		11/4/80	153605	O		9/25/80
153915	non O		11/4/80	153860	non O		10/28/80
153973	O		11/11/80	153599	O		9/25/80
154024	non O		11/18/80	153862	non O		10/28/80
154005	O		11/13/80	153867	non O		10/28/80
154009	non O		11/18/80	153612	O		9/25/80
154001	non O		11/13/80	153858	O		10/28/80
154027	O		11/18/80	153865	O		10/28/80
154001	non O		11/13/80	153864	O		10/28/80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153469	non O	[illegible]	9-9-80	153730	O	[illegible]	10-14-80
153472	O		9-9-80	153675	O		10-2-80
153455	O		9-9-80	153677	non O		10-2-80
153477	non O		9-9-80	153678	O		10-7-80
153472	O		9-9-80	153678	O		10-7-80
153209	O		8-5-80	153674	non O		10-2-80
153607	non O		9-25-80	153033	O		7-10-80
153183	O		8-5-80	153055	non O		7-15-80
153458	O		9-9-80	153026	O		7-10-80
153478	non O		9-9-80	153732	O		10-14-80
153458	O		9-9-80	153671	non O		10-2-80
153468	O		9-9-80	153677	non O		10-2-80
153469	non O		9-9-80	153667	O		10-2-80
153468	O		9-9-80	153671	non O		10-2-80
153475	non O		9-9-80	153676	O		10-2-80
153487	O		9-11-80	153675	O		10-2-80
153465	O		9-9-80	153663	O		10-2-80
153269	O		8-14-80	153665	O		10-2-80
153466	non O		9-9-80	153944	non O		11-6-80
153463	O		9-9-80	153947	non O		11-6-80
153477	non O		9-9-80	153949	O		11-6-80
153455	O		9-9-80	153945	O		11-6-80
153463	O		9-9-80	153947	non O		11-6-80
153466	non O		9-9-80	153949	O		11-6-80
153465	O		9-9-80	153905	O		11-4-80
153471	O		9-9-80	153676	O		10-2-80
153471	O		9-9-80	153732	O		10-14-80
153478	non O		9-9-80	153921	O		11-4-80
153269	O		8-14-80	153145	O		11-6-80
153487	O		9-11-80	153897	O		10-30-80
153475	non O		9-9-80	153922	non O		11-4-80
153318	non O		8-19-80	153897	O		10-30-80
153318	non O		8-19-80	153922	non O		11-4-80
153315	O		8-19-80	153944	non O		11-6-80
153315	O		8-19-80	153953	non O		11-6-80
153314	O		8-19-80	153954	O		11-6-80
153314	O		8-19-80	73340	non O		1-22-80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
73353	O	FH Nat	1-24-80	73960	non O	FH Nat	7-1-80
73340	non O		1-22-80	73951	O		7-1-80
73339	O		1-22-80	73961	non O		7-1-80
153954	O		11-6-80	73945	O		7-1-80
153976	non O		11-11-80	73949	non O		7-1-80
153961	non O		11-11-80	73962	O		7-1-80
153961	non O		11-11-80	73930	O		6-26-80
153953	non O		11-6-80	73950	O		7-1-80
73936	O		6-26-80	73965	O		7-1-80
73342	non O		1-22-80	73954	O		7-1-80
73342	non O		1-22-80	73965	O		7-1-80
73353	O		1-24-80	73950	O		7-1-80
73339	O		1-22-80	73954	O		7-1-80
73346	O		1-22-80	153998	non O		11-13-80
73346	O		1-22-80	153720	O		10-9-80
73345	O		1-22-80	153717	O		10-9-80
73345	O		1-22-80	153998	non O		11-13-80
73914	O		6-24-80	153716	O		10-9-80
73907	O		6-26-80	153997	non O		11-13-80
73932	non O		6-26-80	153984	non O		11-13-80
73932	non O		6-26-80	153984	non O		11-13-80
73936	O		6-26-80	153720	O		10-9-80
73935	O		6-26-80	153796	O		10-21-80
73919	O		6-26-80	153797	O		10-21-80
73919	O		6-26-80	153838	O		10-23-80
73960	non O		7-1-80	153832	O		10-23-80
73932	non O		6-26-80	153801	O		10-21-80
73927	O		6-26-80	153797	O		10-21-80
73937	non O		6-26-80	153830	O		10-21-80
73927	O		6-26-80	153830	O		10-21-80
73918	non O		6-26-80	153834	non O		10-23-80
73927	O		6-26-80	153832	O		10-23-80
73914	O		6-24-80	153644	O		10-7-80
73930	O		6-26-80	153705	O		10-1-80
73962	O		7-1-80	153548	non O		9-18-80
73948	O		7-1-80	153540	O		9-18-80
73951	O	↓	7-1-80	153535	O	↓	9-16-80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153864	O	[illegible]	10/28/80	153701	non O	[illegible]	10/7/80
153865	O		10/28/80	153177	O		7/31/80
153867	non O		10/28/80	153191	O		8/5/80
153603	non O		9/25/80	153177	O		7/31/80
153605	O		9/25/80	153207	O		8/5/80
153614	non O		9/25/80	153191	O		8/5/80
153842	non O		10/23/80	153203	O		8/5/80
153054	O		7/15/80	153203	O		8/5/80
153054	O		7/15/80	153697	non O		10/7/80
153057	O		7/15/80	153698	O		10/7/80
153055	non O		7/15/80	153211	O		8/5/80
153040	O		7/15/80	153208	O		8/5/80
153033	O		7/10/80	153192	non O		8/5/80
153039	non O		7/15/80	153697	non O		10/7/80
153026	O		7/10/80	153208	O		8/5/80
153029	O		7/10/80	153206	O		8/5/80
153056	O		7/15/80	153206	O		8/5/80
153031	O		7/10/80	153207	O		8/5/80
153057	O		7/15/80	153692	O		10/7/80
153040	O		7/15/80	153693	O		10/7/80
153049	O		7/15/80	133551	O		9/18/80
153701	non O		10/7/80	153561	non O		9/18/80
153703	non O		10/7/80	153537	O		9/18/80
153709	non O		10/9/80	153540	O		9/18/80
153707	O		10/9/80	153537	O		9/18/80
153706	O		10/9/80	153692	O		10/7/80
153704	non O		10/7/80	153693	O		10/7/80
153703	non O		10/7/80	153594	O		9-25-80
153706	O		10/9/80	153746	O		10-12-80
153714	non O		10/9/80	153905	O		11-4-80
153707	O		10/9/80	153772	O		10-16-80
153698	O		10/7/80	153892	non O		10-30-80
153718	non O		10/9/80	153839	non O		10-23-80
153718	non O		10/9/80	153884	O		10-30-80
153709	non O		10/9/80	153745	O		10-14-80
153714	non O		10/9/80	153887	non O		10-30-80
153717	O	↓	10/9/80	153561	non O	↓	9-18-80

(A2) HUMAN CRYOPOOR PLASMA (cont'd):

Donor #	Type	Source	Date Drawn	Donor #	Type	Source	Date Drawn
153547	O		9-25-80	153514	non-O		9-16-80
153544	O		9-25-80	153527	non-O		9-16-80
154082	non-O		11-20-80	153548	non-O		9-18-80
154083	non-O		11-20-80	153696	non-O		10-7-80
154084	O		11-25-80	153696	non-O		10-7-80
154083	non-O		11-20-80	153539	O		9-18-80
154080	non-O		11-25-80				
154076	O		11-25-80				
154033	non-O		11-18-80				
154058	O		11-20-80				
154076	O		11-25-80				
154058	O		11-20-80				
154084	O		11-25-80				
153596	non-O		9-25-80				
153593	O		9-25-80				
153591	non-O		9-23-80				
073850	O		6-17-80				
073841	non-O		6-17-80				
154035	O		11-18-80				
154052	O		11-20-80				
154033	non-O		11-18-80				
154083	non-O		11-20-80				
073841	non-O		6-17-80				
154054	non-O		11-20-80				
153551	O		9-18-80				
153559	non-O		9-18-80				
153527	non-O		9-16-80				
153550	O		9-18-80				
153535	O		9-18-80				
153552	O		9-18-80				
153549	O		9-18-80				
154035	O		11-18-80				
153550	O		9-18-80				
153547	O		9-18-80				
153544	non-O		9-18-80				
153552	O		9-18-80				
153539	O		9-18-80				

A Complete Concept in Consultation

CAPSULE LABORATORIES

A Division of Economics Laboratory, Inc.
Research & Development Center
840 Sibley Memorial Hwy., St. Paul, MN 55118 • (612) 457-4926

October 19, 1981

Sterility Audit Report to: University of Minnesota
Date Received: October 2, 1981
Date Tested: October 2, 1981
Sample Description: Anti-Botulinal Toxins IVBG-1-A
Date Bottled: October 2, 1981
Reference: 21 CFR Part 610.12

Sample Description	Media*	Results
#1	SCD	Negative
	TG	Negative
#2	SCD	Negative
	TG	Negative
#3	SCD	Negative
	TG	Negative
#4	SCD	Negative
	TG	Negative
#5	SCD	Negative
	TG	Negative
#6	SCD	Negative
	TG	Negative
#7	SCD	Negative
	TG	Negative
#8	SCD	Negative
	TG	Negative
#9	SCD	Negative
	TG	Negative
#10	SCD	Negative
	TG	Negative
#11	SCD	Negative
	TG	Negative

Sample Description	Media	Results
#12	SCD	Negative
	TG	Negative
#13	SCD	Negative
	TG	Negative
#14	SCD	Negative
	TG	Negative
#15	SCD	Negative
	TG	Negative
#16	SCD	Negative
	TG	Negative
#17	SCD	Negative
	TG	Negative
#18	SCD	Negative
	TG	Negative
#19	SCD	Negative
	TG	Negative
#20	SCD	Negative
	TG	Negative

* SCD - Soybean Casein Digest 20-25°C incubation for 14 days
TG - Fluid Thioglycollate 30-35°C incubation for 14 days

CAPSULE LABORATORIES

Lynn Long

Lynn Long
Microbiologist

LL:pal

TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECIEVED: 12/13/1982

DATE TEST ENDED: 12/27/1982

SAMPLE DESCRIPTION: BOTULISM IMMUNE GLOBULIN

LOG NUMBER: 14445

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA TG	MEDIA SCD	INCUBATION DAYS	INCUBATION TEMP.	POSITIVE TG	POSITIVE SCD
IVBG-1B	#1	2	1	1	14	32/22	0	0
IVBG-1B	#2	2	1	1	14	32/22	0	0
IVBG-1B	#3	2	1	1	14	32/22	0	0
IVBG-1B	#4	2	1	1	14	32/22	0	0
IVBG-1B	#5	2	1	1	14	32/22	0	0
IVBG-1B	#6	2	1	1	14	32/22	0	0
IVBG-1B	#7	2	1	1	14	32/22	0	0
IVBG-1B	#8	2	1	1	14	32/22	0	0
IVBG-1B	#9	2	1	1	14	32/22	0	0
IVBG-1B	#10	2	1	1	14	32/22	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long

Lynn Long
Microbiologist

CAPSULE LABORATORIES

TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECIEVED: 12/13/1982

DATE TEST ENDED: 12/27/1982

SAMPLE DESCRIPTION: BOTULISM IMMUNE GLOBULIN

LOG NUMBER: 14445

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA TG	MEDIA SCD	INCUBATION DAYS	INCUBATION TEMP.	POSITIVE TG	POSITIVE SCD
IVBG-1B	#11	2	1	1	14	32/22	0	0
IVBG-1B	#12	2	1	1	14	32/22	0	0
IVBG-1B	#13	2	1	1	14	32/22	0	0
IVBG-1B	#14	2	1	1	14	32/22	0	0
IVBG-1B	#15	2	1	1	14	32/22	0	0
IVBG-1B	#16	2	1	1	14	32/22	0	0
IVBG-1B	#17	2	1	1	14	32/22	0	0
IVBG-1B	#18	2	1	1	14	32/22	0	0
IVBG-1B	#19	2	1	1	14	32/22	0	0
IVBG-1B	#20	2	1	1	14	32/22	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long

Lynn Long
Microbiologist

CAPSULE LABORATORIES

TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECIEVED: 7/30/1982

DATE TEST ENDED: 8/17/1982

SAMPLE DESCRIPTION: BIOLOGICS

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA TG	MEDIA SCD	INCUBATION DAYS	INCUBATION TEMP.	CONTAMINATED TG	CONTAMINATED SCD
IVBG-2A	#1	2	1	1	14	32/22	0	0
IVBG-2A	#2	2	1	1	14	32/22	0	0
IVBG-2A	#3	2	1	1	14	32/22	0	0
IVBG-2A	#4	2	1	1	14	32/22	0	0
IVBG-2A	#5	2	1	1	14	32/22	0	0
IVBG-2A	#6	2	1	1	14	32/22	0	0
IVBG-2A	#7	2	1	1	14	32/22	0	0
IVBG-2A	#8	2	1	1	14	32/22	0	0
IVBG-2A	#9	2	1	1	14	32/22	0	0
IVBG-2A	#10	2	1	1	14	32/22	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long

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TECHNICAL DATA

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SAMPLE DESCRIPTION: BIOLOGICS

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA TG	MEDIA SCD	INCUBATION DAYS	INCUBATION TEMP.	CONTAMINATED TG	CONTAMINATED SCD
IVBG-2A	#11	2	1	1	14	22/32	0	0
IVBG-2A	#12	2	1	1	14	22/32	0	0
IVBG-2A	#13	2	1	1	14	22/32	0	0
IVBG-2A	#14	2	1	1	14	22/32	0	0
IVBG-2A	#15	2	1	1	14	22/32	0	0
IVBG-2A	#16	2	1	1	14	22/32	0	0
IVBG-2A	#17	2	1	1	14	22/32	0	0
IVBG-2A	#18	2	1	1	14	22/32	0	0
IVBG-2A	#19	2	1	1	14	22/32	0	0
IVBG-2A	#20	2	1	1	14	22/32	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long

Lynn Long
Microbiologist

TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECIEVED: 8/? '1982

DATE TEST ENDED: 9/10/1982

SAMPLE DESCRIPTION: ANTIBOTULINAL TOXIN

LOG NUMBER: 14067

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA TG	MEDIA SCD	INCUBATION DAYS	INCUBATION TEMP.	POSITIVE TG	POSITIVE SCD
IVBG-2B	#1	2	1	1	14	32/22	0	0
IVBG-2B	#2	2	1	1	14	32/22	0	0
IVBG-2B	#3	2	1	1	14	32/22	0	0
IVBG-2B	#4	2	1	1	14	32/22	0	0
IVBG-2B	#5	2	1	1	14	32/22	0	0
IVBG-2B	#6	2	1	1	14	32/22	0	0
IVBG-2B	#7	2	1	1	14	32/22	0	0
IVBG-2B	#8	2	1	1	14	32/22	0	0
IVBG-2B	#9	2	1	1	14	32/22	0	0
IVBG-2B	#10	2	1	1	14	32/22	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long

Lynn Long
Microbiologist

TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECIEVED: 8/27/1982

DATE TEST ENDED: 9/10/1982

SAMPLE DESCRIPTION: ANTIBOTULINAL TOXIN

LOG NUMBER: 14067

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA TG	MEDIA SCD	INCUBATION DAYS	INCUBATION TEMP.	POSITIVE TG	POSITIVE SCD
IVBG-2B	#11	2	1	1	14	32/22	0	0
IVBG-2B	#12	2	1	1	14	32/22	0	0
IVBG-2B	#13	2	1	1	14	32/22	0	0
IVBG-2B	#14	2	1	1	14	32/22	0	0
IVBG-2B	#15	2	1	1	14	32/22	0	0
IVBG-2B	#16	2	1	1	14	32/22	0	0
IVBG-2B	#17	2	1	1	14	32/22	0	0
IVBG-2B	#18	2	1	1	14	32/22	0	0
IVBG-2B	#19	2	1	1	14	32/22	0	0
IVBG-2B	#20	2	1	1	14	32/22	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long

Lynn Long
Microbiologist

CAPSULE LABORATORIES

Sample Tested: IVBG-1A

Date Bottled: 10/2/82

PYROGEN TEST RECORD SHEET

Times Tested: 1) X 2) ____ 3) ____

Date Conditioned: 1/5/83 By: J.P.

Date Tested: 1/5/83 By: J.P.

Rabbit #	cc Injected	Pre	30'	60'	120'	180'	Avg. Rise	High Deviation
10	2.03	38.65	38.9	39.1	39.0	38.85	.313	.45
11	2.11	38.65	38.85	38.85	38.9	38.9	.225	.25
12	2.22	38.85	39.0	39.0	39.05	39.1	.188	.25

Group Avg. Rise: .242

Group Avg. of Highest Deviation: .317

Passed: X Failed: ____

Comments: ____

Appendix E

Sample Tested: IVBG-1B (rebottled)

Date Bottled: 12/9/82

PYROGEN TEST RECORD SHEET

Times Tested: 1) X 2) ____ 3) ____

Date Conditioned: 12/13/82 By: J.P.

Date Tested: 12/13/82 By: J.P.

Rabbit #	cc Injected	Pre	30'	60'	120'	180'	Avg. Rise	High Deviation
11	2.05	39.25	39.2	39.15	39.1	39.1	0	0
12	2.11	39.05	39.35	39.35	39.25	39.0	.25	.3
13	2.11	39.25	39.2	38.9	38.9	38.95	0	0

Group Avg. Rise: .083

Group Avg. of Highest Deviation: .10

Passed: X Failed: ____

Comments: ____

Sample Tested: IVBG-2A

Date Bottled: 7/30/82

PYROGEN TEST RECORD SHEET

Times Tested: 1) X 2) _____ 3) _____

Date Conditioned: 1/18/83 By: J.P.

Date Tested: 1/19/83 By: J.P.

Rabbit #	cc Injected	Pre	30'	60'	120'	180'	Avg. Rise	High Deviation
11	1.91	39.0	39.15	39.4	39.5	39.4	.363	.5
12	1.83	39.35	39.25	39.65	39.75	39.65	.225	.4
13	1.66	39.1	39.05	39.4	39.6	39.25	.25	.5

Group Avg. Rise: .279

Group Avg. of Highest Deviation: .467

Passed: X Failed: _____

Comments: BR#10 - failed - temperature rise of 0.8°C

Sample Tested: IVBG-2B

Date Bottled: 8/27/82

PYROGEN TEST RECORD SHEET

Times Tested: 1) X 2) ____ 3) ____

Date Conditioned: 1/19/83 By: J.P.

Date Tested: 1/20/83 By: P.V.

Rabbit #	cc Injected	Pre	30'	60'	120'	180'	Avg. Rise	High Deviation
10	1.94	39.0	39.1	39.1	39.15	39.15	.125	.15
11	1.97	39.35	39.5	39.45	39.4	39.6	.138	.25
12	1.91	39.3	39.4	39.4	39.3	39.3	.05	.1
13	1.69	38.7	38.85	39.1	39.1	39.1	.337	.4

Group Avg. Rise: .163

Group Avg. of Highest Deviation: .225

Passed: X Failed: ____

Comments: ____

SAFETY TEST

LOT # IVBG-1-A

GUINEA PIGS 5.0cc I.P. each

Received 9-29-81

Test started 10-2-81 By PV Test ended 10-9-81 By PV

Description	Pre injection weight	Weight end of test	
#1 Brown/White	270 grams	335 grams	(Pass) or Fail
#2 Brown	295 grams	330 grams	(Pass) or Fail

MICE .5cc I.P. each

Type SW ♀

Test started 10-2-81 By PV Test ended 10-9-81 By PV

	Pre injection weight	Weight at end of test	
#1 Not clipped	19.1 grams	25.6 grams	(Pass) or Fail
#2 Clipped	20.0 grams	24.1 grams	(Pass) or Fail

SAFETY TEST

LOT # IVBG-1B (Rebottled 12/9/82)

GUINEA PIGS 5.0cc I.P. each

Received 12/17/82

Test started 12/21/82 By P.V Test ended 12/29/82* By P.V.

Description	Pre injection weight	Weight end of test	
#1 Brown	265 grams	330 grams	Pass or Fail
#2 Black	230 grams	255 grams	Pass or Fail

MICE .5cc I.P. each

Type SW ♀

Test started 12/15/82 By P.V. Test ended 12/22/82 By P.V.

	Pre injection weight	Weight at end of test	
#1 Not clipped	20.4 grams	21.1 grams	Pass or Fail
#2 Clipped	19.8 grams	21.0 grams	Pass or Fail

*The animals were not weighed on 12/28/82 (Day 7) due to a snowstorm that resulted in the University closing.

SAFETY TEST

LOT # IVBG-2A

GUINEA PIGS 5.0cc I.P. each

Received 7-22-82

Test started 7-30-82 By P.V. Test ended 8-06-82 By L.N.

Description	Pre injection weight	Weight end of test	
#1 Brown/White	360 gm	400 gm	(Pass) or Fail
#2 White	275 gm	310 gm	(Pass) or Fail

MICE .5cc I.P. each

Type SW ♀

Test started 8-05-82 By P.V. Test ended 8-12-82 By L.N.

	Pre injection weight	Weight at end of test	
#1 Not clipped	17.9 gm	19.4 gm	(Pass) or Fail
#2 Clipped	17.5 gm	19.3 gm	(Pass) or Fail

SAFETY TEST

LOT # IVBG-2B

GUINEA PIGS 5.0cc I.P. each

Received 8-27-82

Test started 8-27-82 By P.V. & L.N. Test ended 9-03-82 By P.V.

Description	Pre injection weight	Weight end of test	
#1 Brown	265 gm	295 gm	(Pass) or Fail
#2 Brown/White	250 gm	295 gm	(Pass) or Fail

MICE .5cc I.P. each

Type SW ♀

Test started 9-02-82 By P.V. Test ended 9-09-82 By P.V.

	Pre injection weight	Weight at end of test	
#1 Not clipped	20.8 gm	21.3 gm	(Pass) or Fail
#2 Clipped	21.0 gm	23.0 gm	(Pass) or Fail

UNIVERSITY OF MINNESOTA HOSPITALS
DEPARTMENT OF LABORATORY MEDICINE AND PATHOLOGY
BOX 198 MAYO MEMORIAL BUILDING
420 DELAWARE STREET S.E.
MINNEAPOLIS, MINNESOTA 55455

NAME	SURGERY, DEPT	DATE REPORTED	16:23 8 OCT 19
CHART	IVBG-1A	UH CHART	0193-00000331
ACCOUNT	06201743	ADMIT DATE	10/ 6/1981

DR. RICHARD CONDI
BUDGET # 0697-5986-02
BOX 383, MAYO

COLLECTION DATE AND TIME	ACCESSION NUMBER	TEST NAMES	SPECIMEN TYPE RESULTS UNITS	REFERENCE VALUES SEX AGE RANGE
10/ 6/81 1450	5467-6	HEPATITIS B	BLOOD	
		HBSAG	NEG	M/F 0-127 YR NEG
		ANTI-HBS	POS	M/F 0-127 YR NEG

UNIVERSITY OF MINNESOTA HOSPITALS AND CLINICS

SUMMARY OF LABORATORY RESULTS

PATIENT [illegible] BED LOCATION SP0193/SP DATE: 5/19/8
DISCHARGE IVBG-1B TIME: 19:42
[illegible] SEX: AGE 0 DOCTOR: PAGE 1

VIROLOGY

BLOOD BANK

	HEPATITIS B				
BLOOD	[illegible]	[illegible]	ANTI-HBC	ANTI-HBE	ANTI-HBS
NORMALS	NEG	NEG	NEG	NEG	NEG
UNITS					
MAY 12 1200	NEG	NEG	POS	POS	POS
	(5/19/ 8)				

PATIENT I.D. BED LOCATION: DATE:

UNIVERSITY OF MINNESOTA HOSPITALS AND CLINICS

SUMMARY OF LABORATORY RESULTS

PATIENT IVBG, 2A
DISCHARGE
193-0000000-1 SEX. AGE 0 DOCTOR:

BED LOCATION SP0193/SP

DATE 8/ 8/82
TIME 12:19
PAGE: 1

VIROLOGY

BLOOD BANK

	HEPATITIS B		
BLOOD	HBSAG	ANTI-HBC	ANTI-HBS
NORMALS	NEG	NEG	NEG
UNITS			
AUG 4 1140	NEG	POS	POS
	(8/ 5/82)		

UNIVERSITY OF MINNESOTA HOSPITALS AND CLINICS

SUMMARY OF LABORATORY RESULTS

PATIENT: IVBG, 2P
DISCHARGE:
I.D. 193-7000002-1 SEX: AGE 0 DOCTOR:

BED LOCATION SP0193/SP DATE: 9/ 3/8
TIME: 19:27
PAGE: 1

VIROLOGY

BLOOD BANK

	HEPATITIS B				
BLOOD	HBSAG	HBEAG	ANTI-HBC	ANTI-HBE	ANTI-HBS
NORMALS	NEG	NEG	NEG	NEG	NEG
UNITS					
AUG 31 1520	NEG	NEG	POS	POS	POS
	(9/ 3/82)				

not acts which require such pharmacies to register.

(b) Practitioners who are licensed by law to prescribe or administer drugs and who manufacture blood products solely for use in the course of their professional practice.

(c) Persons who manufacture blood products which are not for sale, rather, are solely for use in research, teaching, or analysis, including laboratory samples.

(d) Carriers, by reason of their receipt, carriage, holding, or delivery of blood products in the usual course of business as carriers.

(e) Persons who engage solely in the manufacture of in vitro diagnostic blood products and reagents not subject to licensing under section 351 of the Public Health Service Act (42 U.S.C. 262).

PART 610—GENERAL BIOLOGICAL PRODUCTS STANDARDS

Subpart A—Release Requirements

Sec.

Subpart B—General Provisions

Subpart C—Standard Preparations and Limits of Potency

Subpart D—Mycoplasma

Subpart E—Hepatitis Requirements

Subpart F—Dating Period Limitations

Subpart G—Labeling Standards

AUTHORITY: Sec. 215, 58 Stat. 690, as amended; 42 U.S.C. 216. Sec. 351, 58 Stat. 702, as amended; 42 U.S.C. 262, unless otherwise noted.

SOURCE: 38 FR 32056, Nov. 20, 1973, unless otherwise noted.

CROSS REFERENCES. For U.S. Customs Service regulations relating to viruses, serums, and toxins, see 19 CFR 12.21–12.23. For U.S. Postal Service regulations relating to the admissibility to the United States mails see 39 CFR Parts 124 and 125, esp. § 125.2.

Subpart A—Release Requirements

§ 610.1 Tests prior to release required for each lot.

No lot of any licensed product shall be released by the manufacturer prior to the completion of tests for conformity with standards applicable to such product. Each applicable test shall be made on each lot after completion of all processes of manufacture which may affect compliance with the standard to which the test applies. The results of all tests performed shall be considered in determining whether or not the test results meet the test objective, except that a test result may be disregarded when it is established that the test is invalid due to causes unrelated to the product.

§ 610.2 Requests for samples and protocols; official release.

(a) *General.* Samples of any lot of any licensed product, except for radioactive biological products, together with the protocols showing results of applicable tests, may at any time be required to be sent to the Director, Bureau of Biologics. Upon notification by the Director, Bureau of Biologics, a manufacturer shall not distribute a lot of a product until the lot is released by the Director, Bureau of Biologics: *Provided*, That the Director, Bureau of Biologics, shall not issue such notification except when deemed necessary for the safety, purity, or potency of the product.

(b) *Radioactive biological products.* Samples of any lot of a radioactive biological product, as defined in § 600.3(ee) of this chapter, together with the protocols showing results of applicable tests, may at any time be required to be sent to the Food and Drug Administration for official release. Upon notification by the

Director, Bureau of Drugs, a manufacturer shall not distribute a lot of a radioactive biological product until the lot is released by the Director, Bureau of Drugs: *Provided*, That the Director, Bureau of Drugs shall not issue such notification except when deemed necessary for the safety, purity, or potency of the product.

[40 FR 31318, July 25, 1975]

Subpart B—General Provisions

§ 610.10 Potency.

Tests for potency shall consist of either in vitro or in vivo tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition in § 600.3(s) of this chapter.

§ 610.11 General safety.

A general safety test for the detection of extraneous toxic contaminants shall be performed on biological products intended for administration to humans. The general safety test is required in addition to other specific tests prescribed in the additional standards for individual products in this subchapter, except that, the test need not be performed on those products listed in paragraph (g) of this section. The general safety test shall be performed as specified in this section, unless: Modification is prescribed in the additional standards for specific products, or variation is approved as an amendment to the product license under paragraph (f) of this section.

(a) *Product to be tested.* The general safety test shall be conducted upon a representative sample of the product in the final container from every final filling of each lot of the product. If any product is processed further after filling, such as by freeze-drying, sterilization, or heat treatment, the test shall be conducted upon a sample from each filling of each drying chamber run, sterilization chamber, or heat treatment bath.

(b) *Test animals.* Only overtly healthy guinea pigs weighing less than 400 grams each and mice weighing less than 22 grams each shall be used. The animals shall not have been used previously for any test purpose.

(c) *Procedure.* The duration of the general safety test shall be 7 days for both species, except that a longer period may be established for specific products in accordance with paragraph (f) of this section. Once the manufacturer has established a specific duration of the test period for a specific product, it cannot be varied subsequently, except, in accordance with paragraph (f) of this section. Each test animal shall be weighed and the individual weights recorded immediately prior to injection and on the last day of the test. Each animal shall be observed every working day. Any animal response including any which is not specific for or expected from the product and which may indicate a difference in its quality shall be recorded on the day such response is observed. The test product shall be administered as follows:

(1) *Liquid product or freeze-dried product which has been reconstituted as directed on the label.* Inject intraperitoneally 0.5 milliliter of the liquid product or the reconstituted product into each of at least two mice, and 5.0 milliliters of the liquid product or the reconstituted product into each of at least two guinea pigs.

(2) *Freeze-dried product for which the volume of reconstitution is not indicated on the label.* The route of administration, test dose, and diluent shall be as approved by the Director, Bureau of Biologics, in accordance with paragraph (f) of this section. Administer the test product as approved on at least two mice and at least two guinea pigs.

(3) *Nonliquid products other than freeze-dried product.* The route of administration, test dose, and diluent shall be as approved by the Director, Bureau of Biologics, in accordance with paragraph (f) of this section. Dissolve or grind and suspend the product in the approved diluent. Administer the test product as approved on at least two mice and at least two guinea pigs.

(d) *Test requirements.* A safety test is satisfactory if all animals meet all of the following requirements:

(1) They survive the test period.

(2) They do not exhibit any response which is not specific for or expected from the product and which may indicate a difference in its quality.

(3) They weigh no less at the end of the test period than at the time of injection.

(e) *Repeat tests*—(1) *First repeat test.* If a filling fails to meet the requirements of paragraph (d) of this section in the initial test, a repeat test may be conducted on the species which failed the initial test, as prescribed in paragraph

(c) of this section. The filling is satisfactory only if each retest animal meets the requirements prescribed in paragraph (d) of this section.

(2) *Second repeat test.* If a filling fails to meet the requirements of the first repeat test, a second repeat test may be conducted on the species which failed the test: *Provided,* That 50 percent of the total number of animals in that species has survived the initial and first repeat tests. The second repeat test shall be conducted as prescribed in paragraph (c) of this section, except that the number of animals shall be twice that used in the first repeat test. The filling is satisfactory only if each second repeat test animal meets the requirements prescribed in paragraph (d) of this section.

(f) *Test variations.* Variations in the general safety test, such as test dose, route of administration, or duration of the test period may be offered as an amendment to the product license and must receive written approval by the Director, Bureau of Biologics, Food and Drug Administration. Approval will be given only if the license amendment provides substantial evidence demonstrating that the proposed test variation will assure sensitivity equal to or greater than the test prescribed in this section.

(g) *Exceptions.* The test prescribed in this section need not be performed for Whole Blood (Human), Red Blood Cells (Human), Cryoprecipitated Antihemophilic Factor (Human), Platelet Concentrate (Human), or Single Donor Plasma (Human).

[41 FR 10891, Mar. 15, 1976]

§ 610.11a Inactivated influenza vaccine, general safety test.

For inactivated influenza vaccine, the general safety test shall be conducted in the manner indicated in § 610.11 of this chapter except that, with reference to guinea pigs, the test shall be satisfied if the product provides satisfactory results using either the subcutaneous or intraperitoneal injection of 5.0 milliliters of inactivated influenza vaccine into each guinea pig. The requirements for general safety for inactivated influenza vaccine shall not be considered to be satisfied unless each lot of influenza vaccine is assayed for endotoxin in comparison to a reference preparation provided by the Food and Drug Administration, and such lot is found to contain no more endotoxin than the reference preparation.

[39 FR 40016, Nov. 13, 1974]

§ 610.12 Sterility.

Except as provided in paragraphs (f) and (g) of this section, the sterility of each lot of each product shall be demonstrated by the performance of the tests prescribed in paragraphs (a) and (b) of this section for both bulk and final container material.

(a) *The test.* Bulk material shall be tested separately from final container material and material from each final container shall be tested in individual test vessels as follows:

(1) *Using Fluid Thioglycollate Medium*—(i) *Bulk and final container material.* The volume of product, as required by paragraph (d) of this section (hereinafter referred to also as the "inoculum"), from samples of both bulk and final container material, shall be inoculated into test vessels of Fluid Thioglycollate Medium. The inoculum and medium shall be mixed thoroughly and incubated at a temperature of 30° to 32° C. for a test period of no less than 14 days and examined visually for evidence of growth on the third, fourth, or fifth day and on the seventh or eighth day and on the last day of the test period. Results of each examination shall be recorded. If the inoculum renders the medium turbid so that the absence of growth cannot be determined reliably by visual examination, portions of this turbid medium in amounts of no less than 1.0 ml. shall be transferred on the third, fourth, or fifth day of incubation, from each of the test vessels and inoculated into additional vessels of medium. The material in the additional vessels shall be incubated at a temperature of 30° to 32° C. for no less than 14 days. Notwithstanding such transfer of material, examination of the original vessels shall be continued as prescribed above. The additional test vessels shall be examined visually for evidence of growth on the third, fourth, or fifth day of incubation and on the seventh or eighth day and on the last day of the incubation period. If growth appears, repeat tests may be performed as prescribed in paragraph (b) of this section and interpreted as specified in paragraph (c) of this section.

(ii) *Final container material containing a mercurial preservative.* In addition to the test prescribed in subparagraph (1) (i) of this paragraph, final container

material containing a mercurial preservative shall be tested using Fluid Thioglycollate Medium following the procedures prescribed in such subparagraph, except that the incubation shall be at a temperature of 20° to 25° C.

(2) *Using Soybean-Casein Digest Medium.* Except for products containing a mercurial preservative, a test shall be made on final container material, following the procedures prescribed in subparagraph (1)(i) of this paragraph, except that the medium shall be Soybean-Casein Digest Medium and the incubation shall be at a temperature of 20° to 25° C.

(b) *Repeat tests.* If growth appears in any of the test media during testing of either bulk or final container material, the test may be repeated to rule out faulty test procedures as follows:

(1) *Repeat bulk test.* The volume of inoculum to be used for the repeat bulk test shall be as prescribed in paragraph (d)(1) of this section. The repeat test shall be performed using the procedure prescribed in paragraph (a)(1)(i) of this section.

(2) *First repeat final container test.* The number of test samples and the volumes of product used for the first repeat test shall be as prescribed in paragraph (d)(2) of this section. For products that do not contain a mercurial preservative, the repeat test shall be performed, using both Fluid Thioglycollate Medium and Soybean-Casein Digest Medium, following the procedures prescribed in paragraphs (a)(1)(i) and (a)(2), respectively, of this section. If the product contains a mercurial preservative, the repeat test shall be performed using Fluid Thioglycollate Medium and the procedures prescribed in paragraph (a)(1)(i) and (ii) of this section.

(3) *Second repeat final container test.* If growth appears in any of the first repeat final container tests, all tests of the first repeat final container test shall be repeated, provided there was no evidence of growth in any test of the bulk material. The test samples used for the second repeat final container test shall be twice the number used for the first repeat final container test.

(c) *Interpretation of test results.* The results of all tests performed on a lot shall be considered in determining whether or not the lot meets the requirements for sterility, except that tests may be excluded when demonstrated by adequate controls to be invalid. The lot meets the test requirements if no growth appears in the tests prescribed in paragraph (a) of this section. If repeat tests are performed, the lot meets the test requirements if no growth appears in the tests prescribed in paragraph (b)(2) or (3) of this section, whichever is applicable.

(d) *Test samples and volumes.*—(1) *Bulk.* Each sample for the bulk sterility test shall be representative of the bulk material and the volume tested shall be no less than 10 ml. (Note exceptions in paragraph (g) of this section.)

(2) *Final containers.* The sample used for each test medium or each incubation temperature of a test medium for the final container and first repeat final container test shall be no less than 20 final containers from each filling of each lot, selected to represent all stages of filling from the bulk vessel. If the amount of material in the final container is 1.0 milliliter or less, the entire contents shall be tested. If the amount of material in the final container is more than 1.0 milliliter, the volume tested shall be the largest single dose recommended by the manufacturer or 1.0 milliliter, whichever is larger, but no more than 10 milliliters of material or the entire contents from a single final container need be tested. If more than 2 filling machines, each with either single or multiple filling stations, are used for filling one lot, no less than 10 filled containers shall be tested from each filling machine for each test medium or each incubation temperature condition, but no more than 100 containers of each lot need be tested. The items tested shall be representative of each filling assembly and shall be selected to represent all stages of the filling operation. (Note exceptions in paragraph (g) of this section.)

(e) *Culture medium.*—(1) *Formulae.* (i) The formula for Fluid Thioglycollate Medium is as follows:

FLUID THIOGLYCOLLATE MEDIUM

l-cystine	0.5 Gm.
Sodium chloride	2.5 Gm.
Dextrose ($C_6H_{12}O_6 \cdot H_2O$)	5.5 Gm.
Granular agar (less than 15% moisture by weight).	0.75 Gm.
Yeast extract (water-soluble)	5.0 Gm.
Pancreatic digest of casein	15.0 Gm.
Purified water	1,000.0 ml.
Sodium thioglycollate (or thioglycolic acid—0.3 ml.).	0.5 Gm.
Resazurin (0.10% solution, freshly prepared).	1.0 ml.

pH after sterilization 7.1±0.2

(ii) The formula for Soybean-Casein Digest Medium is as follows:

SOYBEAN-CASEIN DIGEST MEDIUM

Pancreatic Digest of Casein	17.0 Gm.
Papaic Digest of Soybean Meal.	3.0 Gm.
Sodium Chloride	5.0 Gm.
Dibasic Potassium Phosphate	2.5 Gm.
Dextrose ($C_6H_{12}O_6H_2O$)	2.5 Gm.
Purified water	1,000.0 ml.
pH after sterilization 7.3±0.2	

(2) *Culture media requirements*—(i) *Growth promoting qualities.* Each lot of dehydrated medium bearing the manufacturer's identifying number, or each lot of medium prepared from basic ingredients, shall be tested for its growth-promoting qualities using not more than 100 organisms of two or more strains of microorganisms that are exacting in their nutritive and aerobic-anaerobic requirements.

(ii) *Conditions of medium and design of test vessels.* A medium shall not be used if the extent of evaporation affects its fluidity, nor shall it be reused in a sterility test. Fluid Thioglycollate Medium shall not be used if more than the upper one-third has acquired a pink color. The medium may be restored once by heating on a steam bath or in free-flowing steam until the pink color disappears. The design of the test vessel for Fluid Thioglycollate Medium shall be such as is shown to provide favorable aerobic and anaerobic growth of microorganisms throughout the test period.

(iii) *Ratio of the inoculum to culture medium.* The ratio of the inoculum to the volume of the culture medium resulting in a dilution of the product that is not bacteriostatic or fungistatic shall be determined for each product, except for those tested by membrane filtration. Vessels of the product-medium mixture(s) and control vessels of the medium shall be inoculated with dilutions of cultures of bacteria or fungi which are sensitive to the product being tested, and incubated at the appropriate temperature for no less than 7 days. Inhibitors or neutralizers of preservatives may be considered in determining the proper ratio.

(f) *Membrane filtration.* Bulk and final container material of products containing oil or products in water insoluble ointments shall be tested for sterility using the membrane filtration procedure set forth in The United States Pharmacopeia [1] (18th Revision, 1970), section entitled "Membrane Filtration," pages 853–854, except that (1) the test samples shall conform with paragraph (d) of this section, (2) the temperature of incubation for the test using Fluid Thioglycollate Medium shall be 30° to 32° C. and (3) in addition, for products containing a mercurial preservative, the product shall be tested in a second test using Fluid Thioglycollate Medium incubated at 20° to 25° C. in lieu of the test in Soybean-Casein Digest Medium. Such Membrane Filtration section is hereby incorporated by reference and deemed published herein. The United States Pharmacopeia is available at most medical and public libraries and copies of the pertinent section will be provided to any manufacturer affected by the provisions of this subchapter upon request to the Director, Bureau of Biologics or the appropriate Information Center Offices listed in 45 CFR Part 5. In addition, an official historic file of the material incorporated by reference is maintained in the office of the Director, Bureau of Biologics.

(g) *Exceptions.* Bulk and final container material shall be tested for sterility as described above in this section, except as follows:

(1) *Different sterility tests prescribed.* When different sterility tests are prescribed for a product in this subchapter.

(2) *Alternate incubation temperatures.* Two tests may be performed, in all respects as prescribed in paragraph (a)(1)(i) of this section, one test using an incubation temperature of 18° to 22° C., the other test using an incubation temperature of 35° to 37° C., in lieu of performing one test using an incubation temperature of 30° to 32° C.

(3) *Different tests equal or superior.* A different test (such as membrane filtration as set forth in paragraph (f) of this section) may be performed provided that prior to the performance of such test a manufacturer submits data which the Commissioner of Food and Drugs, finds adequate to establish that the different test is equal or superior to the tests described in paragraphs (a) and (b) of this section in detecting contamination and makes the finding a matter of official record.

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[1] Copies may be obtained from: United States Pharmacopeial Convention, Inc., 12601 Twinbrook Parkway, Rockville, MD 20852.

(4) *Test precluded or not required.* The tests prescribed in this section need not be performed for Whole Blood (Human), Cryoprecipitated Antihemophilic Factor (Human), Platelet Concentrate (Human), Leukocyte Typing Serum, Red Blood Cells (Human), Single Donor Plasma (Human), Source Plasma (Human), Smallpox Vaccine and other similar products concerning which the Commissioner of Food and Drugs, finds that the mode of administration, the method of preparation or the special nature of the product precludes or does not require a sterility test.

(5) *Number of final containers more than 20, less than 200.* If the number of final containers in the filling is more than 20 or less than 200, the sample shall be no less than 10 percent of the containers.

(6) *Number of final containers—20 or less.* If the number of final containers in a filling is 20 or less, the sample shall be two final containers, or the sample need be no more than one final container, provided (i) the bulk material met the sterility test requirements and (ii) after filling, it is demonstrated by testing a simulated sample that all surfaces to which the product was exposed were free of contaminating microorganisms. The simulated sample shall be prepared by rinsing the filling equipment with sterile 1.0 percent peptone solution, pH 7.1±0.1, which shall be discharged into a final container by the same method used for filling the final containers with the product.

(7) *Samples—large volume of product in final containers.* For Normal Serum Albumin (Human), Plasma Protein Fraction (Human), and Fibrinogen (Human) when the volume of product in the final container is 50 milliliters or more, the final containers selected as the test sample may contain less than the full volume of product in the final containers of the filling from which the sample is taken: *Provided,* That the containers and closures of the sample are identical with those used for the filling to which the test applies, and the sample represents all stages of that filling.

(8) *Diagnostic products not intended for injection.* For diagnostic products not intended for injection, (i) only the Thioglycollate Medium test incubated at 30° to 32° C. is required, (ii) the volume of material for the bulk test shall be no less than 2.0 ml., and (iii) the sample for the final container test shall be no less than three final containers if the total number filled is 100 or less, and if greater, one additional container for each additional 50 containers or fraction thereof, but the sample need be no more than 10 containers.

(9) *Immune globulin preparations.* For immune globulin preparations, the test samples from the bulk material and from each final container need be no more than 2.0 ml.

[38 FR 32056, Nov. 20, 1973, as amended at 40 FR 4304, Jan. 29, 1975; 41 FR 4015, Jan. 28, 1976; 41 FR 10428, Mar. 11, 1976]

§ 610.13 Purity.

Products shall be free from extraneous material except for unavoidable bacteriophage. In addition, products shall be tested as provided in paragraphs (a) and (b) of this section.

(a) *Test for residual moisture.* Each lot of dried product shall be tested for residual moisture and other volatile substances.

(1) *Procedure.* The test for dried products shall consist of measuring the maximum loss of weight in a weighed sample equilibrated over anhydrous P_2O_5 at a pressure of not more than one mm. of mercury, and at a temperature of 20° to 30° C. for as long as it has been established is sufficient to result in a constant weight.

(2) *Test results; standard to be met.* The residual moisture and other volatile substances shall not exceed 1 percent except that, (i) they shall not exceed 1.5 percent for BCG Vaccine; (ii) they shall not exceed 2 percent for Measles Virus Vaccine, Live, Attenuated; Measles-Smallpox Vaccine, Live; Rubella Virus Vaccine, Live; and Antihemophilic Factor (Human); (iii) they shall not exceed 3 percent for Modified Plasma (Bovine); Thrombin; Fibrinogen: Streptokinase; and Streptokinase-Streptodornase; and (iv) they shall not exceed 4.5 percent for Antibody to Hepatitis B Surface Antigen for the Reversed Passive Hemagglutination Test.

(b) *Test for pyrogenic substances.* Each lot of any product intended for use by injection shall be tested for pyrogenic substances by intravenous injection into rabbits as provided in paragraph (b) (1) and (2) of this section: *Provided,* That notwithstanding any other provision of Subchapter F of this chapter, the test for pyrogenic substances is not required for the following products: Products containing formed blood elements; Cry-

oprecipitated Antihemophilic Factor (Human); Single Donor Plasma (Human); Source Plasma (Human); Normal Horse Serum; bacterial, viral, and rickettsial vaccines and antigens; toxoids; toxins; allergenic extracts; venoms; diagnostic substances and trivalent organic arsenicals.

(1) *Test dose.* The test dose for each rabbit shall be at least 3 milliliters per kilogram of body weight of the rabbit and also shall be at least equivalent proportionately, on a body weight basis, to the maximum single human dose recommended, but need not exceed 10 milliliters per kilogram of body weight of the rabbit, except that: (i) Regardless of the human dose recommended, the test dose per kilogram of body weight of each rabbit shall be at least 1 milliliter for Immune globulins derived from human blood and at least 30 milligrams for Fibrinogen (Human); (ii) for Streptokinase, Streptokinase-Streptodornase, Aggregated Radio-Iodinated (I^{131}) Albumin (Human), Radio-Chromated (Cr^{51}) Serum Albumin (Human), Radio-Iodinated (I^{125}) Serum Albumin (Human), and Radio-Iodinated (I^{131}) Serum Albumin (Human), the test dose shall be at least equivalent proportionately, on a body weight basis, to the maximum single human dose recommended.

(2) *Procedure; test results; standards to be met.* Use only overtly healthy mature rabbits. House the rabbits individually in an area of uniform temperature (±3° C), free from disturbances likely to excite them. Rabbits for pyrogen tests may not be used within 2 weeks following their having been given a test sample that was adjudged pyrogenic, and in no case may a rabbit be used more frequently than every 48 hours. Before using a rabbit that has never been used in a pyrogen test, condition it by a sham test that includes all the steps prescribed in paragraph (b)(2) of this section, except injection of the test dose. Perform the test under environmental conditions similar to those under which the rabbits are housed. During the test withhold all food, except water, from the rabbits. Determine the control temperature of each rabbit not more than 40 minutes before injecting the test dose. The control temperature is determined by inserting the temperature measuring device into the rectum of the test rabbit to a depth of not less than 7.5 centimeters and allowing sufficient time to reach a maximum temperature before taking the reading. The control temperature recorded for each rabbit constitutes the temperature from which any subsequent rise following the injection of the material is calculated. If the rectal temperature measuring device is to remain inserted throughout the testing period, restrain the rabbits in a manner that will allow them to assume a natural resting position. In any one test, use only those rabbits whose control temperatures do not deviate more than 1° C from each other, and do not use any rabbits with a temperature exceeding 39.8° C. Warm the test product to approximately 37° C, and inject the test dose into an ear vein of each of three rabbits. Injection of each test dose shall be completed within 10 minutes after the start of administration. Record the temperature of each rabbit at 1, 2, and 3 hours subsequent to the starting time of each injection. The lot meets the requirements for absence of pyrogens if no rabbit shows a rise in temperature of 0.6° C or more above its respective control temperature at any time period, and if the sum of the three rabbits' individual maximum temperature rises does not exceed 1.4° C.

(3) *Retest.* If the lot fails to meet the test requirements prescribed in paragraph (b)(2) of this section, the test may be repeated once using five other rabbits. The temperature rises recorded for all eight rabbits used in testing shall be included in determining whether the requirements are met. The lot meets the requirements for absence of pyrogens if not more than three of the eight rabbits show individual rises in temperature of 0.6° C or more, and if the sum of the eight individual maximum temperature rises does not exceed 3.7° C.

(c) *Different tests equal or superior.* A different test for residual moisture may be performed provided that prior to its performance the manufacturer submits data which the Commissioner of Food and Drugs finds adequate to establish that the different test is equal or superior to the test described in paragraph (a) of this section and makes the finding a matter of official record.

[38 FR 32056, Nov. 20, 1973, as amended at 40 FR 29710, July 15, 1975; 41 FR 10429, Mar. 11, 1976; 41 FR 41424, Sept. 22, 1976]

§ 610.14 Identity.

The contents of a final container of each filling of each lot shall be tested for identity after all labeling operations

shall have been completed. The identity test shall be specific for each product in a manner that will adequately identify it as the product designated on final container and package labels and circulars, and distinguish it from any other product being processed in the same laboratory. Identity may be established either through the physical or chemical characteristics of the product, inspection by macroscopic or microscopic methods, specific cultural tests, or in vitro or in vivo immunological tests.

§ 610.15 Constituent materials.

(a) *Ingredients, preservative, diluents, adjuvants.* All ingredients used in a licensed product, and any diluent provided as an aid in the administration of the product, shall meet generally accepted standards of purity and quality. Any preservative used shall be sufficiently nontoxic so that the amount present in the recommended dose of the product will not be toxic to the recipient, and in the combination used shall not denature the specific substances in the product below the minimum acceptable potency within the dating period when stored at the recommended temperature. Products in multiple dose containers shall contain a preservative, except that a preservative need not be added to Yellow Fever Vaccine, Poliovirus Vaccine, Live, Oral, or to viral vaccines labeled for use with the jet injector, or to dried vaccines when the accompanying diluent contains a preservative. An adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not affect adversely the safety or potency of the product. In no event shall the recommended individual dose of a biological product contain more than 0.85 milligram of aluminum, determined by assay, or more than 1.14 milligrams of aluminum, determined by calculation on the basis of the amount of aluminum compound added.

(b) *Extraneous protein; cell culture produced vaccines.* Extraneous protein known to be capable of producing allergenic effects in human subjects shall not be added to a final virus medium of cell culture produced vaccines intended for injection. If serum is used at any stage, its calculated concentration in the final medium shall not exceed 1:1,000,000.

(c) *Antibiotics.* A minimum concentration of antibiotics, other than penicillin, may be added to the production substrate of viral vaccines.

§ 610.16 Total solids in serums.

Except as otherwise provided by regulation, no liquid serum or antitoxin shall contain more than 20 percent total solids.

§ 610.17 Permissible combinations.

Licensed products may not be combined with other licensed products either therapeutic, prophylactic or diagnostic, except as a license is obtained for the combined product. Licensed products may not be combined with nonlicensable therapeutic, prophylactic, or diagnostic substances except as a license is obtained for such combination.

§ 610.18 Cultures.

(a) *Storage and maintenance.* Cultures used in the manufacture of products shall be stored in a secure and orderly manner, at a temperature and by a method that will retain the initial characteristics of the organisms and insure freedom from contamination and deterioration.

(b) *Identity and verification.* Each culture shall be clearly identified as to source strain. A complete identification of the strain shall be made for each new stock culture preparation. Primary and subsequent seed lots shall be identified by lot number and date of preparation. Periodic tests shall be performed as often as necessary to verify the integrity of the strain characteristics and freedom from extraneous organisms. Results of all periodic tests for verification of cultures and determination of freedom from extraneous organisms shall be recorded and retained.

Subpart C—Standard Preparations and Limits of Potency

§ 610.20 Standard preparations.

Standard preparations made available by the Bureau of Biologics shall be applied in testing, as follows:

(a) *Potency standards.* Potency standards shall be applied in testing for potency all forms of the following:

ANTIBODIES

Botulism Antitoxin, Type A.
Botulism Antitoxin, Type B.
Botulism Antitoxin, Type E.
Diphtheria Antitoxin.
Histolyticus Antitoxin.
Oedematiens Antitoxin.
Perfringens Antitoxin.

Antipertussis Serum.
Antirabies Serum.
Sordellii Antitoxin.
Staphylococcus Antitoxin.
Tetanus Antitoxin.
Vibrion Septique Antitoxin.

ANTIGENS

Cholera Vaccine, Inaba serotype.
Cholera Vaccine, Ogawa serotype.
Diphtheria Toxin for Schick Test.
Pertussis Vaccine.
Tuberculin, Old.
Tuberculin, Purified Protein Derivative.
Typhoid Vaccine.

BLOOD DERIVATIVE

Thrombin.

(b) *Opacity standard.* The U.S. Opacity Standard shall be applied in estimating the bacterial concentration of all bacterial vaccines. The assigned value of the standard when observed visually is 10 units. The assigned value of the standard when observed with a photometer is (i) 10 units when the wavelength of the filter is 530 millimicrons, (ii) 10.6 units when the wavelength of the filter is 650 millimicrons, and (iii) 9 units when the wavelength of the filter is 420 millimicrons.

[38 FR 32056, Nov. 20, 1973, as amended at 41 FR 10429, Mar. 11, 1976; 41 FR 18295, May 3, 1976]

§ 610.21 Limits of potency.

The potency of the following products shall be not less than that set forth below and products dispensed in the dried state shall represent liquid products having the stated limitations.

ANTIBODIES

Diphtheria Antitoxin, 500 units per milliliter.
Tetanus Antitoxin, 400 units per milliliter.
Tetanus Immune Globulin (Human), 50 immunizing dose.

ANTIGENS

Cholera Vaccine, 8 units each of Inaba and Ogawa serotype antigens per milliliter.
Pertussis Vaccine, 12 units per total human units of tetanus antitoxin per milliliter.
Typhoid Vaccine, 8 units per milliliter.

[41 FR 10429, Mar. 11, 1976, as amended at 41 FR 18295, May 3, 1976]

Subpart D—Mycoplasma

§ 610.30 Test for *Mycoplasma*.

Except as provided otherwise in this subchapter, prior to clarification or filtration in the case of live virus vaccines produced from in vitro living cell cultures, and prior to inactivation in the case of inactivated virus vaccines produced from such living cell cultures, each virus harvest pool and control fluid pool shall be tested for the presence of *Mycoplasma*, as follows:

Samples of the virus for this test shall be stored either (1) between 2° and 8° C. for no longer than 24 hours, or (2) at −20° C. or lower if stored for longer than 24 hours. The test shall be performed on samples of the viral harvest pool and on control fluid pool obtained at the time of viral harvest, as follows: No less than 2.0 ml. of each sample shall be inoculated in evenly distributed amounts over the surface of no less than 10 plates of at least two agar media. No less than 1.0 ml. of sample shall be inoculated into each of four tubes containing 10 ml. of a semisolid broth medium. The media shall be such as have been shown to be capable of detecting known *Mycoplasma* and each test shall include control cultures of at least two known strains of *Mycoplasma*, one of which must be *M. pneumoniae*. One half of the plates and two tubes of broth shall be incubated aerobically at 36° C. ±1° C. and the remaining plates and tubes shall be incubated anaerobically at 36° C. ±1° C. in an environment of 5–10 percent CO_2 in N_2. Aerobic incubation shall be for a period of no less than 14 days and the broth in the two tubes shall be tested after 3 days and 14 days, at which times 0.5 ml. of broth from each of the two tubes shall be combined and subinoculated on to no less than 4 additional plates and incubated aerobically. Anaerobic incubation shall be for no less than 14 days and the broth in the two tubes shall be tested after 3 days and 14 days, at which times 0.5 ml. of broth from each of the two tubes shall be combined and subinoculated on to no less than four additional plates and incubated anaerobically. All inoculated plates shall be incubated for no less than 14 days, at which time observation for growth of *Mycoplasma* shall be made at a magnification of no less than 300×. If the Dienes Methylene Blue-Azure dye or an equivalent staining procedure is used, no less than a one square cm. plug of the agar shall be excised from the inoculated area and examined for the presence of *Mycoplasma*. The presence of the *Mycoplasma* shall be determined by comparison of the growth obtained from the test samples with that of the control cultures, with respect to typical colonial and microscopic morphology. The virus pool is satisfactory for vaccine manufacture if none of the tests on the samples show evidence of the presence of *Mycoplasma*.

Subpart E—Hepatitis Requirements

§ 610.40 Test for hepatitis B surface antigen.

(a) *Test sensitivity.* Each donation of blood, plasma, or serum to be used in preparing a biological product shall be

tested for the presence of hepatitis B surface antigen by a method of sufficient sensitivity to detect all sera labeled A, (A), B, (B), and C in the Reference Hepatitis B Surface Antigen Panel distributed by the Bureau of Biologics; except that, in emergency situations, a test method of sufficient sensitivity to detect all sera labeled A, (A), and B in the Reference Hepatitis B Surface Antigen Panel may be used and, in dire emergency situations, blood and blood products may be issued without any HB_sAg testing, provided that a test otherwise required by this paragraph is performed as soon as possible after issuance of the blood and blood product.

(b) *Procedures.* Only Antibody to Hepatitis B Surface Antigen licensed under this subchapter shall be used in performing the test and the test method(s) used shall be that for which the antibody product is specifically designed to be effective as recommended by the manufacturer in the package insert. The sample of blood, plasma, or serum to be tested shall have been taken from the donor at the time of donation of that unit. The test need not be performed on the day of the withdrawal of the sample. If the radioimmunoassay method is used, it must be performed in one of the following ways:

(1) The complete test is performed at the collection facility.

(2) The test is performed at the collection facility up to the point of counting the radioactivity of the samples, which counting, thereafter, is performed at another facility by personnel from the collection facility or by personnel from the counting facility.

(3) The complete test is performed by the personnel at an establishment licensed to manufacture blood or blood derivatives under section 351(a) of the Public Health Service Act (42 U.S.C. 262(a)), or by a clinical laboratory which meets the standards of the Clinical Laboratories Improvement Act of 1967 (CLIA) (42 U.S.C. 263a), provided the establishment or the clinical laboratory is qualified to perform radioimmunoassay testing for the presence of hepatitis B surface antigen. Except for emergencies, blood, plasma or serum shall not be used as a biological product, or issued for use in manufacturing a biological product, until the hepatitis B surface antigen testing is completed and the written results are in the possession of the collection facility.

(c) *Materials in storage.* All blood, plasma, or serum in storage which has not been tested for the presence of the hepatitis B surface antigen shall be tested as required in paragraphs (a) and (b) of this section before use as a biological product, or before use in the manufacture of a biological product. All blood, plasma, or serum in storage which has been tested for the presence of the hepatitis B surface antigen by a method of second generation sensitivity may be used as a biological product or in manufacture of a biological product, provided it is used on or before March 15, 1976.

(d) *Restrictions on use.* (1) *Injectable biological products.* Blood, plasma, or serum that is reactive when tested for hepatitis B surface antigen shall not be used in manufacturing injectable biological products.

(2) *Licensed in vitro diagnostic biological products.* Blood, plasma or serum that is reactive when tested for hepatitis B surface antigen may be used in manufacturing licensed in vitro diagnostic biological products: *Provided,* That (i) the final product cannot be prepared from blood, plasma, or serum that is nonreactive when tested for hepatitis B surface antigen, due either to the nature or to the scarcity of the final product; (ii) the label of the source blood, plasma, or serum conspicuously states that it is reactive when tested for hepatitis B surface antigen and may transmit viral hepatitis; (iii) the package label of the licensed in vitro diagnostic biological product prepared from such blood, plasma, or serum conspicuously states that the product was prepared from material that was reactive when tested for the hepatitis B surface antigen and may transmit viral hepatitis; (iv) at the time of shipment, the Director, Bureau of Biologics, Food and Drug Administration, is notified in writing of each shipment of source blood, plasma, or serum, signifying the kind and amount of source material shipped, the name and address of the consignee, the date of shipment, and the manner in which the source material was labeled. In the case of repetitive shipments of source material for use in manufacturing positive controls for hepatitis B surface antigen testing, the information prescribed in this paragraph (d)(2)(iv) shall be sent to the Director, Bureau of Biologics, on October 1 and

April 1 of each year beginning October 1, 1975.

(3) *Unlicensed in vitro diagnostic biological products.* Blood, plasma, or serum that is reactive when tested for hepatitis B surface antigen may be used in manufacturing unlicensed in vitro diagnostic biological products including clinical chemistry control reagents, *Provided:* That (i) the final product cannot be prepared from blood, plasma, or serum that is nonreactive when tested for hepatitis B surface antigen, due either to the nature or to the scarcity of the final product; (ii) the label of the source blood, plasma, or serum conspicuously states that it is reactive when tested for hepatitis B surface antigen and may transmit viral hepatitis; (iii) the manufacturer of the source blood, plasma, or serum obtains written assurance from the manufacturer(s) of the final unlicensed product that package labels of all unlicensed products will conspicuously state that the product was prepared from blood, plasma or serum that was reactive when tested for the hepatitis B surface antigen and may transmit viral hepatitis, as required by § 809.10(a)(4) of this chapter; (iv) at the time of shipment, the Director, Bureau of Biologics, Food and Drug Administration, is notified in writing of each shipment of source blood, plasma, or serum, signifying the kind and amount of source material shipped, the name and address of the consignee, the date of shipment, and the manner in which such source material was labeled.

(e) *Manufacturing responsibility.* When the radioimmunoassay method for hepatitis B surface antigen testing is performed by personnel other than those of the facility collecting the blood, plasma, or serum, as provided in paragraph (b) of this section, it shall not be considered as divided manufacturing as described in § 610.63, provided the following conditions are met:

(1) The collecting facility has obtained a written agreement that the testing laboratory will permit authorized representatives of the Food and Drug Administration to inspect its testing procedures and facilities during reasonable business hours.

(2) The testing laboratory will participate in any proficiency testing programs undertaken by the Bureau of Biologics, Food and Drug Administration.

[40 FR 29710, July 15, 1975, as amended at 41 FR 6912, Feb. 13, 1976]

§ 610.41 History of hepatitis B surface antigen.

A person testing positive, or known to have previously tested positive, for hepatitis B surface antigen may not serve as a donor of human blood, plasma, or serum to be used in preparing any injectable biological product, except that a person known to have previously tested positive for hepatitis B surface antigen may serve as a source of antibody to hepatitis B surface antigen when such antibody is required for the manufacture of a licensed biological product, provided that the blood of such person meets the requirements of § 610.40.

[40 FR 29710, July 15, 1975]

Subpart F—Dating Period Limitations

§ 610.50 Date of manufacture.

The date of manufacture shall be determined as follows:

(a) For products for which an official standard of potency is prescribed in either § 610.20 or § 610.21, or which are subject to official potency tests, the date of initiation by the manufacturer of the last valid potency test.

(b) For products which are not subject to official potency tests, (1) the date of removal from animals, (2) the date of extraction, (3) the date of solution, or (4) the date of cessation of growth, whichever is applicable.

§ 610.51 Periods of cold storage.

Except as otherwise provided in the regulations of this subchapter, products may be held in cold storage by the manufacturer as follows:

At a temperature not above 5°C.—1 year.
At a temperature not above 0°C.—2 years.

§ 610.52 Dating period.

The dating period for a combination of two or more products shall be no longer than the dating period of the component product with the shortest dating period. The dating period for a product shall begin on the date of manufacture, except that the dating period may begin on the date of issue from the manufacturer's cold storage, provided the product was maintained as prescribed in § 610.51. If held in the manufacturer's cold storage beyond the period prescribed, the dating period shall be reduced by a corresponding period.

Tetanus Antitoxin	*Liquid:* Five years with an initial 20 percent excess of potency. *Dried:* Five years with an initial 10 percent excess of potency.
Tetanus Toxoid	Two years (5° C., one year).
Tetanus Toxoid Adsorbed	Two years (5° C., one year).
Thrombin	Three years.
Trichinella Extract	Eighteen months (5° C., one year).
Tuberculin	*Old, concentrated:* Containing 50 percent glycerin, five years. *Old diluted:* One year. *Purified Protein Derivative, concentrated:* Two years containing 50 percent glycerin (5° C., one year). *Purified Protein Derivative, diluted:* One year. § 610.51 does not apply. *Purified Protein Derivative, dried:* Five years. *Old, dried on multiple puncture device:* Two years, provided labeling recommends storage at no warmer than 30° C. (30° C., one year).
Typhoid Vaccine	Eighteen months (5° C., one year).
Typhus Vaccine	Eighteen months (5° C., one year).
Vaccinia Immune Globulin (Human)	Three years (5° C., three years).
Whole Blood (Human) collected in	(a) ACD solution—Twenty-one days, provided labeling recommends storage between 1° and 10° C. § 610.51 does not apply. (b) Heparin solution—Forty-eight hours, provided labeling recommends storage between 1° and 10° C. § 610.51 does not apply. (c) CPD solution—Twenty-one days, provided labeling recommends storage between 1° and 10° C. § 610.51 does not apply.
Yellow Fever Vaccine	One year, provided labeling recommends storage at no warmer than 5° C. (−20° C., one year).

[39 FR 32[illegible] Nov. 20, 1973, as amended at 39 FR 9660, Mar. 13, 1974; 39 FR 20371, June 10, 1974; 39 FR 27796, Aug. 1, 1974; 40 FR 4304, Jan. 29, 1975; 40 FR 29711, July 15, 1975; 41 FR 10768, Mar. 12, 1976; 41 FR 40101, Sept. 17, 1976; 42 FR 14095, Mar. 15, 1977]

Subpart G—Labeling Standards

§ 610.60 Container label.

(a) *Full label.* The following items shall appear on the label affixed to each container of a product capable of bearing a full label:

(1) The proper name of the product;

(2) The name, address, and license number of manufacturer;

(3) The [illegible] number or other lot identification;

(4) The expiration date;

(5) The recommended individual dose, for multiple dose containers.

(b) *Package label information.* If the container is not enclosed in a package, all the items required for the package label shall appear on the container label.

(c) *Partial label.* If the container is capable of bearing only a partial label, the container shall show as a minimum the name (expressed either as the proper or common name), the lot number or other lot identification and the name of the manufacturer; in addition, for multiple dose containers, the recommended individual dose. Containers bearing partial labels shall be placed in a package which bears all the items required for a package label.

(d) *No container label.* If the container is incapable of bearing any label, the items required for a container label may be omitted, provided the container is placed in a package which bears all the items required for a package label.

(e) *Visual inspection.* When the label has been affixed to the container a sufficient area or the container shall remain uncovered for its full length or circumference to permit inspection of the contents.

§ 610.61 Package label.

The following items shall appear on the label affixed to each package containing a product:

(a) The proper name of the product;

(b) The name, address, and license number of manufacturer;

(c) The lot number or other lot identification;

(d) The expiration date;

(e) The preservative used and its concentration, or if no preservative is used and the absence of a preservative is a safety factor, the words "no preservative";

(f) The number of containers, if more than one;

(g) The amount of product in the container expressed as (1) the number of doses, (2) volume, (3) units of potency, (4) weight, (5) equivalent volume (for dried product to be reconstituted), or (6) such combination of the foregoing as needed for an accurate description of the contents, whichever is applicable;

(h) The recommended storage temperature;

(i) The words "Shake Well", "Do not Freeze" or the equivalent, as well as other instructions, when indicated by the character of the product;

(j) The recommended individual dose if the enclosed container(s) is a multiple-dose container;

(k) The route of administration recommended, or reference to such directions in an enclosed circular;

(l) Known sensitizing substances, or reference to an enclosed circular containing appropriate information;

(m) The type and calculated amount of antibiotics added during manufacture;

(r) The inactive ingredients when a safety factor, or reference to an enclosed circular containing appropriate information;

(o) The adjuvant, if present;

(p) The source of the product when a factor in safe administration;

(q) The identity of each microorganism used in manufacture, and, where applicable, the production medium and the method of inactivation, or reference to an enclosed circular containing appropriate information;

(r) Minimum potency of product expressed in terms of official standard of potency or, if potency is a factor and no U.S. standard of potency has been prescribed, the words "No U.S. standard of potency."

(s) For injectable products prepared from human blood, plasma or serum, a statement that the product was prepared from blood that was nonreactive when tested for hepatitis B surface antigen. In lieu of inclusion on the package label, such information may be included in a circular enclosed with the package.

[38 FR 32056, Nov. 20, 1973, as amended at 40 FR 29711, July 15, 1975]

§ 610.62 Proper name; package label; legible type.

(a) *Position.* The proper name of the product on the package label shall be placed above any trademark or trade name identifying the product and symmetrically arranged with respect to other printing on the label.

(b) *Prominence.* The point size and typeface of the proper name shall be at least as prominent as the point size and typeface used in designating the trademark and trade name. The contrast in color value between the proper name and the background shall be at least as great as the color value between the trademark and trade name and the background. Typography, layout, contrast, and other printing features shall not be used in a manner that will affect adversely the prominence of the proper name.

(c) *Legible type.* All items required to be on the container label and package label shall be in legible type. "Legible type" is type of a size and character which can be read with ease when held in a good light and with normal vision.

§ 610.63 Divided manufacturing responsibility to be shown.

If two or more establishments participate in the manufacture of a product, the name, address, and license number of each must appear on the package label, and on the label of the container if capable of bearing a full label.

§ 610.64 Name of selling agent or distributor.

The name and address of the selling agent or distributor of a product may appear on the label under the designation of "selling agent" or "distributor" provided that the name and address of the manufacturer is given precedence in prominence.

§ 610.65 Products for export.

Labels on packages or containers of products for export may be adapted to meet specific requirements of the regulations of the country to which the product is to be exported provided that in all such cases the minimum label re-

quirements prescribed in § 610.60 are observed.

PART 620—ADDITIONAL STANDARDS FOR BACTERIAL PRODUCTS

Subpart A—Pertussis Vaccine

Subpart B—Typhoid Vaccine

Subpart C—Anthrax Vaccine, Adsorbed

Subpart D—Cholera Vaccine

AUTHORITY: Sec. 215, 58 Stat. 690, as amended; 42 U.S.C. 216. Sec. 351, 58 Stat. 702, as amended; 42 U.S.C. 262, unless otherwise noted.

SOURCE: 38 FR 32064, Nov. 20, 1973, unless otherwise noted.

CROSS REFERENCES.—For U.S. Customs Service regulations relating to viruses, serums, and toxins, see 19 CFR 12.21–12.23. For U.S. Postal Service regulations relating to the admissibility to the United States mails see 39 CFR Parts 124 and 125, esp. § 125.2.

Subpart A—Pertussis Vaccine

§ 620.1 Pertussis Vaccine.

The proper name of this product shall be "Pertussis Vaccine", which shall be an aqueous preparation of either killed whole *Bordetella pertussis* bacteria or a fraction of *Bordetella pertussis* bacteria. The vaccine may be precipitated or adsorbed and may be combined with other antigens.

§ 620.2 Production.

(a) *Propagation of bacteria.* Human blood shall not be used in culture medium for propagating bacteria either for seed or for vaccine. The culture medium for propagating bacteria for vaccine shall not contain ingredients known to be capable of producing allergenic effects in human subjects, except blood or blood products from lower animals other than the horse. When blood or a blood product is used, it shall be removed by washing the harvested bacteria. The bacterial concentrate shall be free of extraneous bacteria, fungi, and yeasts, as demonstrated by microscopic examination and cultural methods.

(b) *Bacterial content.* (1) The opacity of the bacterial concentrate shall be determined in terms of the U.S. Opacity Standard not later than 2 weeks after the harvest of the bacteria and before any treatment capable of altering the opacity of the bacterial concentrate.

(2) The total immunizing dose of a vaccine prepared with whole bacteria shall contain (i) in the case of nonadsorbed vaccine no more bacteria than the equivalent of 60 opacity units and (ii) in the case of adsorbed vaccine no more than the equivalent of 48 opacity units.

(c) *Detoxification.* After removing a sample for purity testing, the bacteria shall be killed and detoxified either (1) by heating, (2) by addition of a chemical agent and appropriate aging, or (3) by any combination of the stated procedures. The procedure used shall be one that has been shown to have no adverse effect on required safety, purity, and potency.

(d) *Preservative.* The vaccine shall contain a preservative.

§ 620.3 U.S. Standard preparations.

(a) The U.S. Standard Pertussis Vaccine shall be used for determining the potency of Pertussis Vaccine.

(b) The U.S. Opacity Standard shall be used in estimating the bacterial content of the vaccine and of the challenge culture.

§ 620.4 Potency test.

The number of protective units of the total human immunizing dose shall be estimated for each lot of vaccine from the results of simultaneous intracerebral mouse protection tests of the vaccine under test and the U.S. Standard Pertussis Vaccine. The potency test shall be performed as follows:

(a) *Mice.* Healthy mice shall be used, all from a single strain and of the same sex, or an equal number of each sex in

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